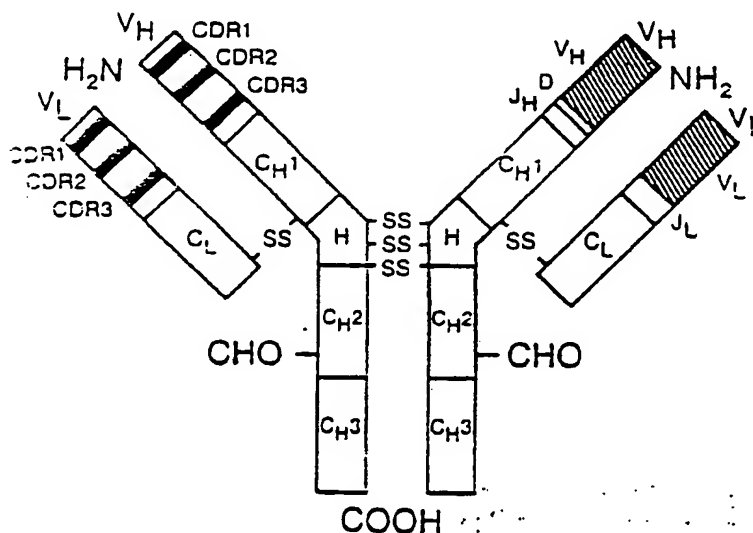




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : A61K 37/04, 39/00, 39/395 A61K 39/44, C07H 15/12, 17/00	A1	(11) International Publication Number: WO 90/04410 (43) International Publication Date: 3 May 1990 (03.05.90)
(21) International Application Number: PCT/US89/04402 (22) International Filing Date: 4 October 1989 (04.10.89) (30) Priority data: 259,943 19 October 1988 (19.10.88) US (71) Applicant: THE DOW CHEMICAL COMPANY [US/US]; 2030 Dow Center, Abbott Road, Midland, MI 48640 (US). (72) Inventors: MEZES, Peter ; 5101 Oakridge Drive, Midland, MI 48640 (US). GOURLIE, Brian ; 3713 Orchard Drive, Midland, MI 48640 (US). RIXON, Mark ; 4110 Castle Drive, Midland, MI 48640 (US). (74) Agent: KIMBLE, Karen, L.; The Dow Chemical Company, P.O. Box 1967, Midland, MI 48641-1967 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent). Published With international search report.

(54) Title: A NOVEL FAMILY OF HIGH AFFINITY, MODIFIED ANTIBODIES FOR CANCER TREATMENT

**(57) Abstract**

This invention concerns a family of chimeric antibodies with high affinities to a high molecular weight, tumor-associated sialylated glycoprotein antigen (TAG72) of human origin. These antibodies have (1) high affinity animal V_H and V_L sequences which mediate TAG-72 binding and (2) human C_H and C_L regions. They are thought to produce significantly fewer side-effects when administered to human patients by virtue of their human C_H and C_L antibody domains. The nucleotide and amino acid sequences of V_H TAG V_H , CC46 V_H , CC49 H , CC83 V_H , and CC92 V_H , and CC49 L , CC83 V_L , and CC92 V_L idiotype sequences are disclosed, as well as *in vivo* methods of treatment and diagnostic assay using these chimeric antibodies.

Best Available Copy

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

A NOVEL FAMILY OF HIGH AFFINITY, MODIFIED
ANTIBODIES FOR CANCER TREATMENT

This invention relates to the field of immunoglobulin production and modifications to naturally occurring antibody amino acid sequences. Specifically,
5 the invention relates to using recombinant DNA techniques to produce chimeric genes and to take advantage of these gene modification techniques to construct chimeric antibodies.

10 Antibodies are specific immunoglobulin (Ig) polypeptides produced by the vertebrate immune system in response to challenges by foreign proteins, glycoproteins, cells or other antigenic foreign
15 substances. The sequence of events which permits the organism to overcome invasion by foreign cells or to rid the system of foreign substances is at least partially understood. An important part of this process is the manufacture of antibodies which bind specifically to a
20 particular foreign substance. The binding specificity of such polypeptides to a particular antigen is highly refined, and the multitude of specificities capable of being generated by the individual vertebrate is
25 remarkable in its complexity and variability. Millions

-2-

of antigens are capable of eliciting antibody responses, each antibody almost exclusively directed to the particular antigen which elicited it.

Two major sources of vertebrate antibodies are presently utilized--generation *in situ* by the mammalian B lymphocytes, and generation in cell culture by B-cell hybrids. Antibodies are generated *in situ* as a result of the differentiation of immature B lymphocytes into plasma cells, which occurs in response to stimulation by specific antigens. In the undifferentiated B cells, the portions of DNA coding for the various regions on the immunoglobulin chains are separated in the genomic DNA. The sequences are assembled sequentially prior to expression. A review of this process has been given by Gough, *Trends in Biochem. Sci.* 6, 203 (1981).

The resulting rearranged gene is capable of expression in the mature B lymphocyte to produce the desired antibody. However, even when a particular mammal is exposed to only a single antigen a uniform population of antibodies does not result. The *in situ* immune response to any particular antigen is defined by the mosaic of responses to the various determinants which are present on the antigen. Each subset of homologous antibodies is contributed by a single population of B cells--hence *in situ* generation of antibodies is "polyclonal".

This limited but inherent heterogeneity has been overcome in numerous particular cases by use of hybridoma technology to create "monoclonal" antibodies in cell cultures by B cell hybridomas [See Kohler and Milstein, C., *Nature* 256, 495-497 (1975)].

-3-

In this process, the relatively short-lived, or mortal, splenocytes or lymphocytes from a mammal which has been injected with antigen are fused with an immortal tumor cell line, thus producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The hybrids thus formed are segregated into single genetic strains by selection, dilution, and regrowth, and each strain thus represents a single genetic line. They therefore produce antibodies which are assured to be homogeneous against a desired antigen. These antibodies, referencing their pure genetic parentage, are called "monoclonal".

Monoclonal antibodies with mono-specificity have greatly influenced immunology, and their usefulness has already been demonstrated in such sciences as biology, pharmacology, chemistry and others. Such monoclonal antibodies have found widespread use not only as diagnostics reagents [see, for example, *Immunology for the 80's*, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, (1981), but also therapy (see, for example, Ritz, J. and Schlossman, S.F., *Blood* 59, 1-11, (1982)].

Monoclonal antibodies produced by hybridomas, while theoretically effective as discussed above and clearly preferable to polyclonal antibodies because of their specificity, suffer from an important disadvantage. In many applications, the use of monoclonal antibodies produced in non-human animals is severely restricted where the monoclonal antibodies are to be used in humans. Repeated injections of a "foreign" antibody in humans, such as a mouse antibody, may lead to harmful hypersensitivity reactions. Such a

non-human derived monoclonal antibody, when injected into humans, causes a anti-nonhuman antibody (ANHA) response. For a discussion of a specific ANHA response caused by using murine-derived antibodies, human anti-mouse antibody (HAMA) response, see Shawler et al.,
5 *Journal of Immunology* 135, 1530-1535 (1985).

It is believed that animal immunoglobulins having human constant regions will generate less of a ANHA response when injected into humans than animal
10 immunoglobulins having nonhuman constant regions. As such, monoclonal antibodies having good binding affinities for selected antigens and having human constant regions are thought to possess great potential utility for immunological diagnosis and therapy of human
15 patients with cancer.

Various attempts have so far been made to manufacture human-derived monoclonal antibodies by using
20 human hybridomas. For example, human-human hybridomas [Olsson, L. et al., *Proc.Natl.Acad.Sci.(USA)*, 77, 5429 (1980)]; human-murine hybridomas [(Schlom, J., et al. (*ibid*) 77, 6841 (1980))] and several other xenogenic hybrid combinations have been prepared. Human
25 monoclonal antibodies have also been produced by transformation of lymphocytes using Epstein-Barr virus. However, such hybridomas may potentially harbor pathogenic human viruses. Alternatively, primary,
antibody producing B cells have been immortalized *in vitro*
30 by transformation with viral DNA. Unfortunately, yields of monoclonal antibodies from human hybridoma cell lines are relatively low (1 µg/mL in human compared to 100 µg/mL in mouse hybridomas), and production costs are high.

-5-

While human immunoglobulins are highly desirable in immunological diagnosis and therapy of human cancer patients, human hybridoma techniques have not yet reached the stage where human monoclonal antibodies with required antigenic specificities can be easily obtained. In addition, for obvious ethical reasons, researchers can not immunize human subjects with selected toxic or otherwise deleterious antigens to generate antibodies against the specific antigen. This imposes great restrictions on immunological diagnosis and therapy of human patients.

The production of human-derived monoclonal antibodies is certainly possible, but is still inefficient in view of its low reproducibility and the other problems noted above. [Additionally, see *Nature* 300, 316-317 (1982)]. Consequently, most monoclonal antibodies are derived from non-human animals.

A monoclonal antibody which reacts with high binding affinity to human tumor antigens, but which is not recognized as a foreign substance by humans is highly desirable. A method to overcome this difficulty is to create artificially an antibody which is very similar to a human antibody and is not recognized as a foreign substance within the human body, i.e., a chimeric, or "humanized" antibody.

Typically in chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to the sequences in antibodies derived from humans. One clear advantage to such chimeric forms is that, for example, the variable regions can conveniently be

-6-

derived from presently known sources using readily available hybridomas of B cells from non-human host organisms in combination with constant regions derived from, for example, human cell preparations. While the specificity of the variable region is not affected by its source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non-human source.

One known human tumor antigen is tumor-associated glycoprotein (TAG72). TAG72 is associated with the surface of certain tumor cells of human origin, specifically the LS174T tumor cell line. LS174T [American Type Culture Collection (herein ATCC) No. CL 188] is a variant of the LS180 (ATCC No. CL 187) colon adenocarcinoma line.

The karyotype of LS174T is similar to that of LS180 with a missing X chromosome in a majority of the cells. Data has been presented as described in Johnson, V.G. et al., *Cancer Res.* 46, 850-857 (1986), to characterize the TAG72 molecule as a mucin. This conclusion is based on the following observations:

- (a) TAG72 has a high molecular weight ($>1 \times 10^6$) as shown by its exclusion from a Sepharose™ CL-4B column;
- (b) the density of TAG72 determined by equilibrium centrifugation in CsCl was 1.45 gm/mL, indicating a heavily glycosylated glycoprotein;
- (c) TAG72 demonstrates a change in migration after neuraminidase digestion, indicating that it is a heavily sialylated molecule with an abundance of O-glycosidically linked oligosaccharides characteristic of mucins;
- (d) blood group antigens commonly found on mucins are found on affinity-purified TAG72; and
- (e) Chondroitinase ABC

-7-

digestion had no effect on TAG72, thus demonstrating that the TAG72 epitope is not expressed on a chondroitin sulfate proteoglycan.

Numerous murine monoclonal antibodies have been developed which have binding specificity for TAG72. One of these monoclonal antibodies, designated B72.3, is a murine IgG1 produced by hybridoma B72.3 (ATCC No. HB-8108). B72.3 is a first generation monoclonal antibody developed using a human breast carcinoma extract as the immunogen (see Colcher, D. et al., *Proc. Natl. Acad. Sci. (USA)* 78, 3199-3203 (1981); and U.S. Patents 4,522,918 and 4,612,282). As used herein, the expression "first generation monoclonal antibody" means a monoclonal antibody produced using, as the immunogen, a crude cell extract.

Other monoclonal antibodies directed against TAG72 are designated "CC" (colon cancer). CC monoclonal antibodies are a family of second generation murine monoclonal antibodies. As used herein, the expression "second generation monoclonal antibody" means a monoclonal antibody produced using, as the immunogen, an antigen purified with a first generation monoclonal antibody. CC monoclonal antibodies were prepared using TAG72 purified with B72.3. A discussion of the method for producing the CC antibodies is set forth in United States Patent Application 7-073,685 (USPA 7-073,685), the application was filed by Schlom et al. on July 15, 1987 and is available to the public from the National Technical Information Service. Because of their relatively good binding affinities to TAG72, the following CC antibodies have been deposited at the ATCC, with restricted access having been requested: CC49 (ATCC No. HB 9459); CC83 (ATCC No. HB 9453); CC46 (ATCC No. HB

-8-

9458); CC92 (ATCC No. HB 9454); CC30 (ATCC NO. HB 9457);
CC11 (ATCC No. 9455); and CC15 (ATCC No. HB 9460).

In the known art, no human antibody has been
isolated which relatively strongly binds to TAG72.

5 Consequently, suitable antibodies must be engineered.

It is known that the function of an Ig molecule
is dependent on its three dimensional structure, which
in turn is dependent on its primary amino acid sequence.

10 Thus, changing the amino acid sequence of an Ig may
adversely affect its activity. Moreover, a change in
the DNA sequence coding for the Ig may affect the
ability of the cell containing the DNA sequence to
express, secrete or assemble Ig.

15

USPA 7-073,685 teaches that the CC antibodies
may be altered into their chimeric form by substituting,
e.g., human constant regions (Fc) domains for mouse
constant regions by recombinant DNA techniques known in
the art. It is believed that the proposals set out in
USPA 7-073,685 did not lead to an actual attempt to
express any chimeric Ig polypeptide chains, nor to
produce Ig activity, nor to secrete and assemble Ig
chains into the desired chimeric Igs.

25

It is therefore not at all clear from the art
that known recombinant DNA techniques will routinely
produce a chimeric animal-human antibody from selected
DNA sources that generate functional chimeric antibodies
which bind specifically to selected human carcinomas and
which reduce the initiation of ANHA side-effects when
injected into humans.

30

Suprisingly, the present invention is able to
meet many of these above mentioned needs and provides a

method for supplying the desired antibodies. For example, the present invention provides a method to fuse genes coding for at least a part of an animal Ig which binds to human carcinomas expressing TAG72 and genes coding for at least part of a human Ig. Also the present invention can provide a method to achieve expression of protein which can be secreted and assembled to give a functional chimeric antibody.

Further the present invention provides an expression vector containing a DNA sequence which encodes antibodies and portions thereof which are directed against TAG72.

The present invention also provides cells transformed with expression vectors containing a DNA sequence which encodes antibodies and portions thereof which are directed against TAG72.

Finally, the present invention provides novel antibodies for use in *in vivo* diagnostic assays; *in vivo* therapy; and radioimmunoguided surgery.

Consequently, this invention concerns an antibody or antibody fragment comprising a variable region having a heavy chain (V_H), said V_H being encoded by a DNA sequence effectively homologous to the V_H TAG germline gene (V_H TAG), wherein the variable region binds to TAG72 at least 25% greater than the variable region of B72.3 binds to TAG72, with the binding affinities of the antibody and B72.3 being measured by the same technique.

This invention also concerns a DNA sequence encoding at least a portion of an antibody heavy chain, said sequence comprising a DNA sequence segment being

-10-

effectively homologous to the V_HαTAG germline gene (V_HαTAG), wherein the DNA sequence segment encodes at least a portion of a heavy chain variable region (V_H).

Further, the invention concerns a DNA sequence
5 comprising:

(A) a sequence segment encoding for a heavy chain, said sequence segment having

(1) a sequence subsegment being effectively
homologous to the V_HαTAG germline gene (V_HαTAG), wherein
10 the DNA sequence segment encodes at least a portion of a V_H, and

(2) a sequence subsegment encoding for at least a portion of a C_H; and

(B) a sequence segment encoding for a light
15 chain, said sequence segment having

(1) a sequence subsegment encoding for at least a portion of an animal light chain variable region (V_L), and

(2) a sequence subsegment encoding for at least a portion of a human light chain constant region (C_L),

wherein the antibody encoded by the DNA sequence binds to TAG72 at least 25% greater than the variable region of B72.3 binds to TAG72, with the
25 binding affinities of the antibody and B72.3 being measured by the same technique.

The invention further includes the
aforementioned antibody alone or conjugated to an
30 imaging marker or therapeutic agent. The invention also includes a composition comprising the aforementioned antibody in unconjugated or conjugated form in a pharmaceutically acceptable, non-toxic, sterile carrier.

-11-

The invention is also directed to a method for *in vivo* diagnosis of cancer which comprises administering to an animal a pharmaceutically effective amount of the aforementioned composition for the *in situ* detection of carcinoma lesions.

5

The invention is also directed to a method for intraoperative therapy which comprises (a) administering to an animal a pharmaceutically effective amount of the aforementioned composition, whereby the tumors are

10 localized, and (b) excision of the localized tumors.

10

Additionally, the invention also concerns process for preparing the various antibodies or antibody fragments, their conjugates, a suitable recombinant

15 expression vehicle, and the insertion into a suitable host. Some of these processes are expressed as follows. A process for preparing an antibody or antibody fragment comprising contacting a V_H region with a V_L region to

20 form a variable region of the antibody or antibody fragment. A process for preparing an antibody or antibody fragment conjugate comprising contacting an antibody or antibody fragment with an imaging marker or therapeutic agent. A process for preparing a

25 recombinant expression vehicle comprising inserting a DNA sequence into an expression vehicle. A process for preparing a transformed host comprising inserting the plasmid into a suitable host.

15

20

25

30

In other aspects, the invention is directed to DNA which encodes the aforementioned antibodies and fragments thereof, as well as expression vectors or plasmids capable of effecting the production of such immunoglobulins in suitable host cells. It includes the

host cells and cell cultures which result from transformation with these vectors.

Description of the Drawings

5 Figure 1 illustrates a basic immunoglobulin structure, with the enzymatic cleavage sites being indicated.

10 Figure 2 illustrates the nucleotide sequences of V_HαTAG V_H, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

 Figure 3 illustrates the amino acid sequences of V_HαTAG V_H, CC46 V_H, CC49 V_H, CC83 V_H and CC92 V_H.

15 Figure 4a illustrates the nucleotide sequence and Figure 4b illustrates the corresponding amino acid sequence of the CC49 V_L.

20 Figure 5a illustrates the nucleotide sequence and Figure 5b illustrates the corresponding amino acid sequence of the CC83 V_L.

25 Figure 6a illustrates the nucleotide sequence and Figure 6b illustrates the corresponding amino acid sequence of the CC92 V_L.

 Figure 7 illustrates the nucleotide sequence of the *Hind* III-*Pst* I fragment isolated from the plasmid pGD1.

30 Figure 8 illustrates the plasmid map of the pBLUESCRIPT SK(-).

 Figure 9 illustrates the plasmid map of the pRL101.

Figure 10 illustrates a restriction enzyme map of the CC49 L chain genomic DNA insert in pRL101.

Figure 11 illustrates the plasmid map of the pRL200.

5

Figure 12 illustrates a restriction enzyme map of the CC83 L chain genomic DNA insert in pRL200.

10

Figure 13 illustrates the nucleotide sequence of the *Eco* RI-*Bam* HI fragment isolated from the plasmid pNP9.

Figure 14 illustrates the plasmid map of the pHH49.

15

Figure 15 illustrates the plasmid map of the pHS83.

20

Figure 16 shows the nucleotide sequence of CC49 V_H , with the underlined segments showing the sequences derived using oligonucleotide primers on mRNA.

25

Figure 17 shows the nucleotide sequence of CC83 V_H , with the underlined segments show the sequences derived using oligonucleotide primers on mRNA.

30

Figure 18 shows the amino acid sequence of CC49 V_H , with the underlined segments show the sequences determined by protein sequencing.

Figure 19 shows the amino acid sequence of CC83 V_H , with the underlined segments show the sequences determined by protein sequencing.

Figure 20 shows the results of a SDS polyacrylamide gel, with the results of PNGase F treatment of CC83 antibody.

Figure 21 illustrates the restriction enzyme map of human gamma 1, gamma 2, gamma 3, and gamma 4.

Figure 22 illustrates the plasmid map of pSV2gpt/R/B.

Figure 23 illustrates the plasmid map of pSV2gpt- γ 1-7.8.

Figure 24 illustrates the plasmid map of pSV2gpt- γ 1-2.3.

Figure 25 illustrates the plasmid map of pSV2gpt- γ 2.

Figure 26 illustrates the plasmid map of pSV2gpt- γ 3.

Figure 27 illustrates the plasmid map of pSV2gpt- γ 4.

Figure 28 illustrates the plasmid map of p49 γ 1-7.8.

Figure 29 illustrates the plasmid map of p49 γ 1-2.3.

Figure 30 illustrates the plasmid map of p49- γ 2.

Figure 31 illustrates the plasmid map of p49- γ 3.

-15-

Figure 32 illustrates the plasmid map of p49-
γ4.

Figure 33 illustrates the plasmid map of p83γ1-
7.8.

5 Figure 34 illustrates the plasmid map of p83γ1-
2.3.

10 Figure 35 illustrates the plasmid map of p83-
γ2.

Figure 36 illustrates the plasmid map of p83-
γ3.

15 Figure 37 illustrates the plasmid map of p83-
γ4.

20 Figure 38 illustrates the overall reaction for
the engineering of hybrid genes based on the method of
Horton et al., *Gene* 77, 61 (1989).

Figures 39A, 39B, and 39C show the
biodistribution and whole body retention of CH44-1.

25 Figures 40A and 40B show the biodistribution
and whole body retention of CH84-1.

30 The immunoglobulin of this invention have been
developed to address the problems of murine monoclonal
antibodies disclosed in the prior art. It is
characterized by having a chimeric structure composed of
a heavy chain variable region encoded by DNA derived
from the VHαTAG.

Definitions

As used herein, "immunoglobulin" refers to a tetramer or aggregate thereof whether or not specific immunoreactive activity is a property. "Antibodies" refers to such assemblies which have significant known specific immunoreactive activity to an antigen, comprising light and heavy chains, with or without covalent linkage between them; "Non-specific immunoglobulin" ("NSI") means those immunoglobulins which do not possess known specificity to an antigen.

The basic immunoglobulin structural unit in vertebrate systems is relatively well understood [Edelman, G.M., *Ann.N.Y.Acad.Sci.*, 190, 5 (1971)]. As seen in Figure 1, the units are composed of two identical light polypeptide chains of molecular weight approximately 23,000 daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the diversity region.

Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, with some subclasses among them. The nature of this chain, as it has a long constant region, determines the "class" of the antibody as IgA, IgD, IgE, IgG or IgM.

Light chains are classified as either kappa (κ) or lambda (λ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains

-17-

are bonded to each other by covalent disulfide linkages when the immunoglobulins are generated either by hybridomas or by B cells. However, if non-covalent association of the chains can be effected in the correct geometry, the aggregate of non-disulfide-linked chains will still be capable of reaction with antigen.

The amino acid sequences run from an N-terminus at the forked edges of the Y to the C-terminus at the bottom of each chain. At the N-terminus is a variable region and at the C-terminus is a constant region.

The terms "constant" and "variable" are used functionally. The variable regions of both light (V_L) and heavy (V_H) chains determine binding recognition and specificity to the antigen. The constant region domains of light (C_L) and heavy (C_H) chains confer important biological properties such as antibody chain association, secretion, transplacental mobility, and complement binding.

The variable region is linked in each chain to the constant region by a linkage linking the V gene sequence and the C gene sequence. The linkage occurs at the genomic level, combining nucleotide sequences via recombination sites. The linking sequence is known currently as a "J" sequence in the light chain gene, which encodes about 12 amino acids, and as a combination of a "D" sequence and a "J" sequence in the heavy chain gene, which together encode approximately 25 amino acids.

"Chimeric antibody" for purposes of this invention refers to an antibody having in the heavy chain a variable region amino acid sequence encoded by a

-18-

nucleotide sequence derived from a murine germline gene and a constant region amino acid sequences encoded by a nucleotide sequence derived from a human gene.

5 However, the present invention is not intended
to be narrowly limited to merely substituting human C
genes sequences encoding immunoglobulin constant regions
for murine C gene sequences encoding immunoglobulin
constant regions. Thus the present invention is not
10 limited to whether or not the fusion point is at the
variable/constant boundary.

Through various techniques, it is now possible
to produce altered chimeric antibodies, composite
chimeric antibodies, and fragmented chimeric antibodies
15 encoded by nucleotide sequences disclosed herein.

"Composite" immunoglobulins comprise
polypeptide variable regions not hitherto found
associated with each other in nature. It is not
20 critical whether any of the above are covalently or non-
covalently aggregated, so long as the aggregation is
capable of selectively reacting with a particular
antigen or antigen family.

25 "Altered antibodies" means antibodies wherein
the amino acid sequences, particularly in the variable
region, has been varied. Because of the relevance of
recombinant DNA techniques to this invention, one need
30 not be confined to the amino acid sequences of
antibodies selected from natural sources; amino acid
sequences of the antibodies can be redesigned to obtain
desired characteristics. The possible variations are
many and range from the changing of just one or a few

-19-

amino acids to the complete redesign of an antibody variable and/or constant region.

Changes in the variable region will be made in order to improve the antigen binding characteristics.

5 Changes in the constant region will, in general, be made in order to improve the cellular process characteristics, such as complement fixation, interaction with membranes, and other effector functions. Alterations, can be made by standard recombinant techniques and also
10 by oligonucleotide-directed mutagenesis techniques [Dalbadie-McFarland, et al. *Proc. Natl. Acad. Sci. (USA)* 79, 6409 (1982)].

"Fragments" of immunoglobulins include segments
15 of proteolytically-cleaved or recombinantly-prepared portions of an antibody molecule that are capable of selectively reacting with a particular antigen or antigen family. Nonlimiting examples of such
20 proteolytic and/or recombinant fragments include "Fab", "F(ab')₂", and "Fab'", with their proteolytic cleavage sites being shown in Figure 1; as well as "Fv" and "V_H". By an "Fv" fragment is meant that portions of light chain variable and heavy chain variable regions are
25 linked together, generally at their carboxy termini. Recombinant techniques for producing Fv fragments are set forth in WO 88/01649, WO 88/06630, WO 88/07085, WO 88/07086 and WO 88/09344. By a "V_H" fragment is meant that the variable region has at least a portion of a
30 heavy chain variable region capable of being used as an antigen binding functionality. The preparation and use of a light chain variable region (V_L) as an antigen binding functionality is set forth in an article entitled "Development of biologically active peptides

-20-

based on antibody structure" by Williams et al. is set forth in *Proc. Natl. Acad. Sci. (USA)* 86, 5537-5541 (1989).

In this invention, "animals" is meant to include bovines, porcine, rodents, and primates, including humans, and others.

"Expression vector" is given a functional definition of any DNA sequence which is capable of effecting expression of a specified DNA code in a suitable host is included in this term. As at present, such vectors are frequently in the form of plasmids; thus "plasmid" and "expression vector" are often used interchangeably. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which may, from time to time, become known in the art.

By "transformation" is meant the introduction of DNA into a recipient host cell that changes the genotype and consequently results in a change in the recipient cell.

"Host cells" refers to cells which have been recombinantly transformed with vectors constructed using recombinant DNA techniques. As defined herein, the antibody or modification thereof produced by a host cell is by virtue of this transformation.

In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of antibody from the "cells" may mean either from spun down whole cells,

or from the cell culture containing both the medium and the suspended cells.

Abbreviations

5 Nucleic acids, amino acids, peptides,
protective groups, active groups and similar moieties,
when abbreviated, are abbreviated according to the
IUPACIUB (Commission on Biological Nomenclature) or the
practice in the fields concerned. The following are
10 examples.

15

20

25

30

-22-

Reagents

EDTA: Ethylenediaminetetraacetic acid

SDS: Sodium dodecylsulfate

5 Nucleic Acids

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid

10 Nitrogenous BasesPurines

A: Adenine

15 G: Guanine

Pyrimidines

T: Thymine

C: Cytosine

U: Uracil

Both DNA and RNA contain long chains of phosphoric acid, a sugar, and nitrogenous bases. DNA is a double stranded helix, wherein the sugar is 2-deoxyribose, whereas RNA is single stranded, wherein the sugar is D-ribose. The four nitrogenous bases which characterize DNA nucleotides are linked in complementary pairs by hydrogen bonds to form the double helix of DNA: adenine is linked to thymine; guanine is linked to cytosine. In RNA, uracil is substituted for thymine in the listed DNA pairs.

30 Amino Acids

Gly: glycine

Ala: alanine

Phe: phenylalanine

Tyr: tyrosine

-23-

Val: valine	Thr: threonine
Leu: leucine	Cys: cysteine
Ile: isoleucine	Met: methionine
5 Ser: serine	Glu: glutamic acid
Asp: aspartic acid	Trp: tryptophan
Lys: lysine	Pro: proline
10 Arg: arginine	Asn: asparagine
His: histidine	Gln: glutamine

Variable Region

15

The DNA encoding the heavy chain consists of a V_H gene sequence, a D_H gene sequence, and a J_H gene sequence. The DNA encoding the light chain consists of a V_L gene sequence, and a J_L gene sequence.

20

 V_H Gene Sequence

The present invention is directed to selected chimeric antibodies having the V_H region encoded by a DNA sequence derived from a germline gene that is specifically reactive against TAG72 ($V_H\alpha$ TAG), the sequence of which is set forth in Figure 2. The chimeric antibodies are selected on the basis of their ability to bind TAG72, namely wherein the variable region binds to TAG72 at least 25 percent greater than the variable region of B72.3 binds to TAG72. Generally, the binding affinities of the chimeric antibody and B72.3 are measured by the same technique. Exemplary techniques for measuring antibody binding affinity are set forth in the following references: Scatchard G.,

25

30

Annals of the N.Y. Acad. of Sciences 51, 660 (1949); Steward, M.W., and Petty, R.E., *Immunology* 23, 881 (1972); Muraro, R., et al., *Cancer Research* 48, 4588 (1988); and Heyman, B., *J. of Immunol. Methods* 68, 193-204 (1984).

5 A skilled artisan will appreciate that, as a result of the present invention, namely the nucleotide sequence of (and amino acid sequences encoded by) the V_HαTAG, the present invention is intended to include
10 effectively homologous nucleotide sequences and corresponding amino acid sequences. "Effectively homologous" refers to identity or near identity of nucleotide or amino acid sequences. Thus, in this
15 disclosure it will be understood that minor sequence variation can exist within homologous sequences and that any sequences exhibiting at least 80% homology are deemed equivalent.

 Homology is expressed at the fraction or
20 percentage of matching bases (or amino acids) after two sequences (possibly of unequal length) have been aligned. The term alignment is used in the sense defined by Sankoff and Kruskal in Chapter One of their book, The Time Warps, String Edits, and Macromolecules:
25 The Theory and Practice of Sequence Comparison, Addison-Wesley, Reading, MA, (1983). Roughly, two sequences are aligned by maximizing the number of matching bases (or amino acids) between the two sequences with the
30 insertion of a minimal number of "blank" or "null" bases into either sequence to bring about the maximum overlap.

 As is understood in the art, nucleotide mismatches can occur at the third or wobble base in the codon without causing amino acid substitutions in the final polypeptide sequence. Also, minor nucleotide

-25-

modifications (e.g., substitutions, insertions or deletions) in certain regions of the gene sequence can be tolerated and considered insignificant whenever such modifications result in changes in amino acid sequence that do not alter functionality of the final product.

5 It has been shown that chemically synthesized copies of whole, or parts of, gene sequences can replace the corresponding regions in the natural gene without loss of gene function.

10 Homologs of specific DNA sequences may be identified by those skilled in the art using the test of cross-hybridization of nucleic acids under conditions of stringency as is well understood in the art [as
15 described in Nucleic Acid Hybridization, Hames and Higgs (eds.), IRL Press, Oxford, UK (1985)]. Given two sequences, algorithms are available for computing their homology: e.g. Needleham and Wunsch, *J. Mol. Biol.*,
48, 443-453 (1970); and Sankoff and Kruskal (cited
20 above) pgs. 23-29. Also, commercial services are available for performing such comparisons, e.g. Intelligenetics, Inc. (Palo Alto, CA).

DH and JH Gene Sequences

25 The DH and JH gene segments exist in various types, although the type of D or J gene segment selected is not critical to the invention. That is the DH and JH may be derived from any animal. Preferred animals
30 include mice and humans. Obviously, human DH and/or JH gene segments are particularly preferred, but the invention is not so limited if a D or J gene segment from another animal species provides an important property, i.e., increased binding to TAG72.

-26-

Exemplary murine D_H and J_H sequences are set forth in "Organization, Structure, and Assembly of Immunoglobulin Heavy Chain Diversity DNA Segments", Kurosawa and Tonegawa, *J.Exp.Med.* 155, 201 (1982); and "Sequences of the Joining Region Genes for Immunoglobulin Heavy Chains and Their Role in Generation of Antibody Diversity", Gough and Bernard, *Proc.Natl.Acad.Sci.(USA)*, 78, 509 (1981).

Exemplary human D_H and J_H sequences are set forth in an article titled "Human Immunoglobulin D Segments encoded in Tandem Multigenic Families" by Siebenlist et al. in *Nature* 294, 631 (1981); and exemplary human J_H sequences are set forth in "Structure of the Human Immunoglobulin μ Locus: Characterization of Embryonic and Rearranged J and D Genes" by Ravetch et al., *Cell*, 27, 583 (1981).

V_L and J_L Gene Sequences

Generally, any V_L and J_L gene sequences may be employed that encodes a portion of a V_L which is complementary to the V_H encoded by a nucleotide sequence effectively homologous to V_HaTAG. By "complementary" means a V_L that binds to the V_H and which yields an antibody variable region having a binding affinity of at least 25% more than B72.3, as measured by any standard technique for measuring binding affinity constants.

The type of V_L and J_L gene segment selected is not critical to the invention. That is the V_L and J_L may be derived from any animal. Preferred animals include mice and humans. Obviously, human V_L and/or J_L gene segments are particularly preferred, but the invention is not so limited if a J_L gene segment from

-27-

another species provides an important property, i.e., increased binding to TAG72.

Murine J_L sequences are set forth in an article titled "The nucleotide Sequence of a 5,5-kilobase DNA Segment Containing the Mouse Kappa Immunoglobulin J and C Region Genes" by Max, et al. in *J.Biol.Chem.* 256, 5116-5120 (1981). Human J_L sequences are set forth in an article titled "Evolution of Human Immunoglobulin K J Region Genes" by Heiter et al. in *The Journal of Biological Chemistry* 357(2), 1516-1522 (1982).

Derivation of Variable Regions

Given the above teachings, it now becomes possible to derive numerous specific embodiments of antibody variable regions within the scope of the present invention, i.e., having effectively homologous V_H sequences to V_HαTAG and binding to TAG72 at least 25% greater than the variable region of B72.3 binds to TAG72, with the binding affinities of the antibody and B72.3 being measured by the same technique. Several possible techniques are set forth below.

Naturally-Produced Variable Regions

In response to an immunogen, TAG72, an immunized animal will expand selected antibody producing B cells. The variable region of antibodies produced by the B cells will be encoded by rearranged germline heavy and light chain DNA. For example, the rearranged germline heavy chain will include the V, D, and J gene segments including the leader sequence, as well as any introns which may be subsequently removed. The light chain coding DNA will include the V and J gene segments

-28-

including the leader sequence, as well as any introns which may be subsequently removed.

Variability may result from somatic mutations occurring in a B cell during productive rearrangement of the $V_H\alpha$ TAG. These somatic mutations are nucleotide changes that may or may not result in an amino acid change that alters the activity toward TAG72 of the productively rearranged V_H .

10 Screening Techniques

Monoclonal or polyclonal antibodies may be screened to determine which of said antibodies selectively bind to TAG72. Such screening may be accomplished by any of a number of well-known procedures, such as solid-phase radioimmunoassay, enzyme-linked immunosorbent assays, rosetting assays and blocking assays. The above-described procedures are well-known in the art.

The nucleotide sequences of encoding variable regions of antibodies produced from the productive rearrangement of the $V_H\alpha$ TAG have now been obtained. In addition to the nucleotide sequence of $V_H\alpha$ TAG, Figure 2 also shows the nucleotide sequences encoding the heavy chain variable regions of CC46, CC49, CC83 and CC92 antibodies, respectively. Figure 3 shows the amino acid sequences of $V_H\alpha$ TAG V_H , CC46 V_H , CC49 V_H , CC83 V_H , and CC92 V_H , corresponding to the nucleotide sequences set forth in Figure 2. A comparison of the nucleotide and amino acid sequences of $V_H\alpha$ TAG V_H , CC46 V_H , CC49 V_H , CC83 V_H and CC92 V_H shows a most striking feature, namely that the chains have extraordinary similarity.

-29-

The relative similarity of the DNA encoding the CC46 V_H, CC49 V_H, CC83 V_H, and CC92 V_H regions, particularly in the 5' flanking segment, proves that those DNA sequences are derived from V_HαTAG. Somatic mutations occurring during productive rearrangement of the V_H region gene to be expressed in a B cell give rise to some nucleotide changes that may or may not result in a homologous amino acid change between two productively rearranged V_HαTAG producing hybridomas.

The nucleotide sequences and corresponding amino acid sequences of CC49 V_L are shown in Figures 4a and 4b, respectively. The nucleotide sequences and corresponding amino acid sequences of CC83 V_L are shown in Figures 5a and 5b, respectively. The nucleotide sequences and corresponding amino acid sequences of CC92 V_L are shown in Figures 6a and 6b, respectively.

Probe Techniques

Other antibodies encoded by DNA derived from V_HαTAG may be derived by using V_HαTAG as a hybridization probe. Generally, a probe made from the DNA or RNA of the V_HαTAG or rearranged genes containing the recombined V_HαTAG could be used by those skilled in the art to find homologous genes in unknown hybridomas. Such homologous antibodies will have a DNA sequence whose mRNA hybridizes with the probe of all or a part of the V_HαTAG germline gene and its flanking regions. By "flanking regions" is meant to include those DNA sequences from the 5' end of the V_HαTAG to the 3' end of the upstream gene, and from 3' end of the V_HαTAG to the 5' end of the downstream gene.

Rationally Synthesized Variable Regions

A yet further approach is the rational synthesis of altered variable regions of the antibodies disclosed herein, as well as antibodies discovered via
5 probing. Such an approach has several potential advantages. Namely, a researcher would not have to screen immunized host animals attempting first to cull those antibodies which bind to TAG and next to cull
10 those antibodies which specifically have V_H regions encoded by DNA derived from $V_H\alpha$ TAG.

Mutagenic Techniques

The V_H and/or V_L gene segments may be
15 "altered" by mutagenesis. Exemplary techniques include the addition, deletion, or nonconservative substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides, provided
20 that the proper reading frame is maintained.

Substitutions, deletions, insertions or any subcombination may be combined to arrive at a final construct. Since there are 64 possible codon sequences but only twenty known amino acids, the genetic code is
25 degenerate in the sense that different codons may yield the same amino acid. However, the code is precise for each amino acid; thus there is at least one codon for each amino acid, i.e., each codon yields a single amino
30 acid and no other. It will be apparent that during translation, the proper reading frame must be maintained in order to obtain the proper amino acid sequence in the polypeptide ultimately produced.

Techniques for additions at predetermined amino acid sites having a known sequence are well known.

-31-

Exemplary techniques include oligonucleotide-mediated, site-directed mutagenesis and polymerase chain reaction.

Techniques for deletions at predetermined amino acid sites having a known sequence are well known.

- 5 Exemplary techniques include oligonucleotide-mediated site-directed mutagenesis and the polymerase chain reaction.

- 10 Techniques for substitutions at predetermined amino acid sites having a known sequence are well known. Exemplary techniques include site-directed mutagenesis, and the polymerase chain reaction technique.

- 15 Oligonucleotide site-directed mutagenesis in essence involves hybridizing an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the
20 oligonucleotide to produce a strand containing the mutation. This technique, in various forms, is described by Zoller, M.J. and Smith, M., *Nuc.Acids Res.* 10, 6487-6500 (1982); Norris, K., Norris, F., Christiansen, L. and Fiii, N., *Nuc.Acids Res.* 11, 5103-5112 (1983); Zoller, M.J. and Smith, M., *DNA* 3, 479-488
25 (1984); Kramer, W., Schughart, K. and Fritz, W. J., *Nuc. Acids Res.* 10, 6475-6485 (1982).

- 30 Polymerase chain reaction (PCR) in essence involves exponentially amplifying DNA in vitro using sequence specified oligonucleotides. The oligonucleotides can incorporate sequence alterations if desired. The polymerase chain reaction technique is described in Mullis and Faloona, *Meth.Enz.* 155, 335-350 (1987). Examples of mutagenesis using PCR are described

-32-

in Higuchi et al., *Nucl. Acids Res.* 16, 7351-7367 (1988),
Ho et al., *Gene* 77, 51-59 (1989), and "Engineering
Hybrid Restriction Genes Without the Use of Restriction
Enzymes: Gene Splicing by Overlap Extension", Horton et
al., *Gene* 77, 61 (1989).

5

Alteration of the antibody variable regions may
be of particular use in the therapeutic use of
monoclonal antibodies. At present, when a chimeric
antibody comprising a complete mouse variable domain is
10 injected into a human, the human body's immune system
recognizes the mouse variable domain, albeit less than a
complete murine antibody, as foreign and produces an
immune response thereto. Thus, on subsequent injections
15 of the mouse antibody or chimeric antibody into the
human, its effectiveness is considerably reduced by the
action of the body's immune system against the foreign
antibody. Consequently, alterations of the murine V_H
and V_L regions may reduce the human immune response to
20 the altered antibody.

Recombinant Techniques

The antibodies may be constructed by
25 recombinant techniques. In other words, because the
nucleotide sequences of various V_H- and V_L-encoding
regions are now provided, a skilled artisan could *in vitro*
produce a complete gene coding for the heavy and light
chain variable regions.

30

The constructed gene may be engineered in which
selected D_H and J_H gene segments are in functional
combination with a selected V_H gene segment, i.e., the
V_HαTAG segment, or the V_H gene segment of CC49 or CC83.

-33-

For example, the constructed heavy chain coding DNA will include D_H and J_H gene sequences which are contiguous with the 3' end of the germline V_H TAG gene segment, thereby completing the CDR3 and framework (FR) 4 of the V_H domain. A leader sequence may be present but may be subsequently removed.

Depending upon the light chain employed, it may also be necessary to provide a constructed light chain coding DNA. Such a DNA gene will comprise a V_L gene segment in functional combination, e.g., contiguous with a J_L gene segment, including the leader sequence which may be subsequently removed. The J_L gene segment will vary depending upon whether the light chain is of the lambda or kappa system. The J region sequence is contiguous with the end of the V_L exon to complete FR 4 of the V_L domain. Such a construction may be carried out by the techniques used to construct the V_H gene.

The constructed gene may be engineered by conventional recombinant techniques for example, to provide a gene insert in a plasmid capable of expression. Thereafter, the plasmids may be expressed in host cells. Exemplary recombinant biological techniques are set forth below.

In providing a fragment encoding either the light chain or heavy chain variable region, it will usually be desirable to include all or a portion of the intron downstream from the J region, particularly where the variable region is derived from the host in which the fused gene is to be expressed. Where the intron is retained, it will be necessary that there be functional splice acceptor and donor sequences at the intron termini. The intron between the J and the constant

-34-

region of the fused gene may be primarily the intron sequence associated with (1) the constant region, (2) the J domain, or (3) portions of each. The last may be a matter of convenience where there is a convenient restriction site in the introns from the two sources.

5 It may be necessary to provide adapters to join the intron to the constant region. In some instances, all or a portion of the intron may be modified by deletion, nucleotide substitution(s) or insertion, to enhance ease of manipulation, expression, or the like. Preferably, a
10 sufficient amount of the intron should be present to contain an enhancer that is functionally active with the naturally-occurring promoter.

15 Alternatively, it may be desirable to have the fused gene free of the intron between the J gene and C gene. Thus, the 3' terminus of the J gene will be adjacent to the 5' terminus of the C gene. One can use an exonuclease and, by employing varying periods of
20 digestion, one can provide for varying 3'-termini, which can then be used for linking to the constant region and selection made for a functional product in a variety of ways; or by splicing with overlap extension using
25 polymerase chain reaction technology, see Horton et al., *supra*. In this case, an artificial promoter, which does not need to be functionally active with an enhancer, will generally be utilized

30 In one preferred embodiment, the genes encoding the V_H and V_L regions may be altered by replacing at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains of the antibody with analogous parts of CDRs from an antibody of different specificity. An exemplary technique replacing the CDRs is taught in European

-35-

Published Patent Application 0 239 400, by Gregory Winter; and in PCT application Wo 88/09344, by Huston et al. In an altered antibody of the present invention, only the CDRs of the antibody will be foreign to a human body, and this should minimize side effects if used for human therapy. However, human and mouse framework regions have characteristic features which distinguish human from mouse framework regions. Thus, an antibody comprised of mouse CDRs in a human framework may well be no more foreign to the body than a genuine human antibody.

The nucleotide sequences corresponding to the V_H amino acid sequences of the V_HαTAG, CC46, CC49, CC83 and CC92, as well as of the CC49, CC83 and CC92 V_L gene segments are provided. Consequently, it is envisaged that the CDRs from the antibodies of the present invention could be grafted onto the framework regions of a human antibody.

Generally, the CDR regions from a human V_H or V_L domain may be replaced by CDRs from the V_H or V_L regions of antibodies of the present invention. Exemplary human antibodies from which the framework portions may be used include human plasmacytoma NEWM, [Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse", *Nature* 321, 522-525 (1986)], publicly available from Dr. Greg Winter; and various other human V_H and V_L genes available from Dr. Terrence Rabbitts, both researchers being from the Medical Research Council, 20 Park Crescent, London, W1N 4AL.

The determination as to what constitutes a CDR and what constitutes a framework region may be made on

the basis of the amino-acid sequences of a selected Ig as indicated in Kabat et al., Sequences of Proteins of Immunological Interest, Fourth Edition (1987), U.S. Dept. of Health and Human Services, NIH.

5 The four framework regions largely adopt a β -sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β -sheet structure.

10 Moreover, not all of the amino-acid residues in the loop regions are solvent accessible and in one case, amino-acid residues in the framework regions are involved in antigen binding. [Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J., *Science* 233, 747
15 -753, (1986)].

 It is also known that the variable regions of the two parts of an antigen binding site are held in the correct orientation by inter-chain, non-covalent
20 interactions. These may involve amino-acid residues within the CDRs.

 Thus, in order to transfer the antigen binding capacity of one variable domain to another, it may not
25 be necessary to replace all of the CDRs with the complete CDRs from the donor variable region. It may be necessary only to transfer those residues which are necessary for the antigen binding site, and this may
30 involve transferring framework region residues as well as CDR residues.

 It is thus clear that merely replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody. However, given the explanations set forth in European Published Patent

-37-

Application 0 239 400, it will be well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing, to obtain a functional altered antibody.

5 Preferably, the variable domains in both the heavy and light chains are altered by at least partial CDR replacement and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class
10 or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different
15 species.

Composite Variable Regions

 Generally, the V gene encoding the V_L is the same V gene which encodes the V_L naturally combined with
20 the V_H of choice. For example, the V gene which encodes the V_L regions of CC49 and CC83 are beneficially used when employing the V gene which encodes the V_H of CC49 and CC83, respectively.

25 Surprisingly, because the V_H regions of the antibodies of the present invention are encoded by V_H genes derived from V_H TAG, composite antibodies may be beneficially formed. In other words, the V_H region of
30 one antibody of the present invention may suitably be combined with the V_L region of another antibody of the present invention. Although the amino acid sequences of the CC49 and CC83 heavy chains are superficially close, it would be expected that a change of a few or even one amino acid would drastically affect the binding function

-38-

of the antibody, i.e., the resultant antibodies are generally presumed to be a non-specific immunoglobulin (NSI), i.e.--lacking in antibody character, (see European Published Patent Application 0 125 023).

5 Quite surprisingly, it has now been found that an antibody having the requisite V_H of this invention, need not be recombined only with a V_L from the same naturally occurring animal antibody. For instance, as
10 set forth in the examples, it is possible to produce a chimeric antibody having a heavy chain with a V_H from CC83 and a light chain with a V_L from CC49, wherein the composite antibody thus formed has a binding specificity 25% greater than the binding affinity of B72.3 to TAG72.

15 Constant Regions

Heavy Chain (C_H) Domain

 The C_H domains may be of various human
20 isotypes, i.e., IgG (e.g., IgG₁, IgG₂, IgG₃ and IgG₄), IgA, IgD, IgM, as well as the various subtypes of the individual groups.

 For a discussion of the human $\gamma 1$, see Ellison
25 et al., "The nucleotide sequence of a human immunoglobulin C-gamma-1 gene", *Nucl. Acid Res* 10, 4071-4079 (1982); Takahashi et al., "Structure of human immunoglobulin gamma genes: Implications for evolution of a gene family", *Cell* 29, 671-679 (1982). For a
30 discussion of the human gamma 2 ($\gamma 2$), see Krawinkel et al., "Comparison of the hinge-coding segments in human immunoglobulin gamma heavy genes and the linkage of the gamma 2 and gamma 4 subclass genes, *EMBO J* 1, 403-407 (1982); Ellison et al., "Linkage and sequence homology of two human immunoglobulin gamma heavy chain constant

region genes, *Proc.Nat.Acad.Sci.(USA)* 79, 1984-1988 (182);
Takahashi et al., *infra*. For a discussion of human gamma
3 (γ 3), see Krawinkel et al. *infra*, and Takahashi et al.,
infra. For a discussion of human gamma 4 (γ 4), see
Ellison et al. "Nucleotide sequence of a human
5 immunoglobulin C-gamma-4 gene, *DNA* 1, 11-18 (1981),
Krawinkel et al. *infra*, and Takahashi et al., *infra*.

For a discussion of the human mu, see Rabbitts
et al., Human Immunoglobulin Heavy Chain Genes:
10 Evolutionary Comparisons of $C\mu$, $C\delta$, and $C\gamma$ genes and
Associated Switch Sequences", *Nucl..Acid Res.* 9, 4509-
45024.

For a discussion of the human alpha, see
15 Flanagan et al., "Mechanisms of Divergence and
Convergence of the Human Immunoglobulin alpha 1 and
alpha 2 Constant Region Gene Sequences", *Cell* 36, 681-688
(1984).

20 For a discussion of the human delta, see White
et al., "Human Immunoglobulin D: Genomic Sequences of
the Delta Heavy Chain", *Science* 228, 733-737 (1985).

25 For a discussion of the human epsilon, see Max
et al., "Duplication and Deletion in the Human
Immunoglobulin ϵ Genes", *Cell* 29, 691-699 (1982).

Light Chain (C_L) Domain

30 The C_L domain may be human kappa (κ) or human
lambda (λ).

For a discussion of the human κ , see "Cloned
Human and Mouse Kappa Immunoglobulin Constant and J

Region Genes Conserve Homology in Functional Segments", Heiter et al., *Cell* 22, 197-207, November (1980)

For a discussion of the human λ , see "Processed Genes: A Dispersed Human Immunoglobulin Gene Bearing Evidence of RNA-Type Processing", Hollis et al., *Nature* 296, 321-325 (1982).

The C_H and/or C_L gene segments may be "altered" by mutagenesis. Exemplary techniques include the addition, deletion, or nonconservative substitution of a limited number of various nucleotides or the conservative substitution of many nucleotides, provided that the proper reading frame is maintained. In addition, entire domains of the protein can be altered, for example, by substituting C_H2 for C_H3. This substitution is made at the DNA level by inserting, deleting or substituting entire exons of sequence.

Construction of Antibodies

Immunizations

The first technique for producing antibodies, whether monoclonal or polyclonal, having V_H regions encoded by DNA derived from V_HαTAG is to immunize a host animal with purified TAG72. Exemplary protocols for immunizing a host animal with TAG72 are set forth in U.S. Patents 4,522,918 and 4,612,282, using a human breast carcinoma extract as the immunogen; and United States Patent Application 7-073,685 (which is available to the public), using TAG72 purified with B72.3 as the immunogen.

Thereafter, monoclonal or polyclonal antibodies produced from the immunization protocol are screened to

-41-

determine which of said antibodies selectively bind to TAG72. Such screening may be accomplished by any of a number of well-known procedures, such as solid-phase radioimmunoassay, enzyme-linked immunosorbent assays, rosetting assays and blocking assays. The above-described procedures are well-known in the art.

Synthesis of Amino Acid Sequences

Immunoglobulins of the present invention can be synthesized from their constituent amino acids. Suitable techniques are the Merrifield solid phase method, as described in *J. Amer. Chem. Soc.* 85, 2149-2154 (1963). This solid phase method for synthesizing sequences of amino acids is also described on pages 1-4 of of a book by Stewart and Young, Solid Phase Peptide Synthesis (W. H. Freeman and Co., San Francisco, 1969).

Construction of DNA

DNA Encoding the V_H and V_L

The DNA encoding the antibody heavy and light chains may be obtained from a variety of sources known to those of ordinary skill in the art, for example, genomic DNA, cDNA, synthetic DNA, or a combination thereof.

Cells coding for the desired sequence may be isolated, and genomic DNA fragmented by one or more restriction enzymes. The genomic DNA may or may not include naturally-occurring introns. The resulting fragments may then be cloned and screened using a heavy chain J region (J_H) probe for the presence of the DNA sequence coding for the polypeptide sequence of interest. DNA fragments isolated by preparative agarose

-42-

gel electrophoresis are ligated. Recombinant plaques of the libraries are screened with a mouse J_H probe.

The DNA may also be obtained from a cDNA library. Messenger RNA coding for heavy or light chain
5 is isolated from a suitable source, either mature B cells or a hybridoma culture, employing standard techniques of RNA isolation, and the use of oligo-dT
cellulose chromatography to segregate the poly-A mRNA. The poly-A mRNA may, further, be fractionated to obtain
10 sequences of sufficient size to code for the amino acid sequences in the light or heavy chain of the desired antibody as necessary.

A cDNA library is then prepared from the
15 mixture of mRNA using a suitable primer, preferably a nucleic acid sequence which is characteristic of the desired cDNA. Such a primer may be synthesized based on the amino acid sequence of the antibody. In the
20 alternative cDNA from unfractionated poly-A mRNA from a cell line producing the desired antibody or poly-dT may also be used. The resulting cDNA is optionally size fractionated on polyacrylamide gel and then extended with, for example, dC residues for annealing with pBR322
25 or other suitable cloning vector which has been cleaved by a suitable restriction enzyme, such as Pst I, and extended with dG residues. Alternative means of forming cloning vectors containing the cDNA using other tails and other cloning vector remainder may, of course, also
30 be used but the foregoing is a standard and preferable choice. A suitable host cell strain, typically *Escherichia coli* (*E.coli*), is transformed with the annealed cloning vectors, and the successful transformants identified by means of, for example, ampicillin or

-43-

tetracycline resistance or other phenotypic characteristics residing on the cloning vector plasmid.

Successful transformants are picked and transferred to microtiter dishes or other support for further growth and preservation. Nitrocellulose filter imprints of these growing cultures are then probed with suitable nucleotide sequences containing bases known to be complementary to desired sequences in the cDNA. Several types of probe may be used, preferably synthetic single stranded DNA sequences labeled by kinasing with γ -³²P ATP. The cells fixed to the nitrocellulose filter are lysed, the DNA denatured, and then fixed before reaction with kinased probe. Clones which successfully hybridize are detected by contact with a photoplate, then plasmids from the growing colonies isolated and sequenced by means known in the art to verify that the desired portions of the gene are present.

The desired gene fragments are excised and tailored to assure appropriate reading frame with the control segments when inserted into suitable expression vectors. Typically, nucleotides are added to the 5' end to include a start signal and a suitably positioned restriction endonuclease site.

Because the inventors have provided the nucleotide sequences of the V_HαTAG, the DNA also may be synthetically synthesized, for example, using an Applied Biosystems™ Model 380A DNA Synthesizer, and constructed by standard techniques.

Finally, an exemplary technique for utilizing combination of the above techniques is by splicing with overlap extension using polymerase chain reaction

-44-

technology, see Horton et al., *supra*. Generally, a synthetically synthesized primer, having a so-called "wagging tail", may be inserted with a selected sequence, for example genomic DNA. Thereafter, the sequences are amplified and spliced together.

5

DNA Encoding the C_H and C_L

The DNA fragment encoding the amino acid sequence of the human constant region may be obtained by screening the chromosomal DNA of cells producing human immunoglobulin.

10

Vectors

The desired DNA fragment may be positioned in a biologically functional expression vehicle which may contain appropriate control sequences not present in the selected DNA fragment. By "biologically functional" is meant that the expression vehicle provides for replication and/or expression in an appropriate host, either by maintenance as an extrachromosomal element or by integration into the host genome. A large number of vectors are available or can be readily prepared, and are well-known to skilled artisans.

15

20

25

A number of plasmids, such as those described in European Published Patent Applns. 0036776, 0048970 and 0051873, have been described which already contain a promoter in reading frame with the gene and compatible with the proposed host cell.

30

The vectors and methods disclosed herein are suitable for use over a wide range of microorganisms, either prokaryotic or eukaryotic, which are susceptible to transformation. The plasmid will be capable of

-45-

replicating in the microorganism, particularly a bacterium.

5 In general, plasmid vectors containing the appropriate promoters, which can be used by the microbial organism for expression of its own protein, also contain control sequences, ribosome binding sites, and transcription termination sites. Generally, the replicon and control sequences which are derived from species compatible with the host cell are used in
10 connection with these hosts.

Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 base pair (bp) sequence extending from the Hind III site
15 toward the *Pvu* II site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the
20 host cell systems.

Finally, the plasmid should desirably have a gene, a marker gene, that is capable of providing a phenotypical property which allows for selection of host
25 cells containing the expression vector. Particularly useful is a gene that provides for survival selection. Survival selection can be achieved by providing resistance to a growth inhibiting substance or providing
30 a growth factor capability to a bacterium deficient in such capability.

In general, prokaryotes are preferred. For example, pBR322 a plasmid derived from an *E. coli* species [Bolivar, et al., *Gene* 2, 95 (1977)] is particularly

-46-

useful. pBR322 contains genes for ampicillin and tetracycline resistance and thus provides an easy means for identifying transformed cells.

While these prokaryotes are the most commonly used, other microbial strains which may be used include *E. coli* strains such as *E. coli* B, *E. coli* K12 strain 294 (ATCC No. 31446) and *E. coli* X1776 (ATCC No. 31537), *E. coli* W3110 (F⁻, γ^- , prototrophic, ATCC No. 27325), bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species may be used. These examples are intended to be illustrative only.

In addition to prokaryotes, eukaryotic microbes may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains are commonly available.

For expression in *Saccharomyces*, the plasmid YRp7, for example, [Stinchcomb, et al., *Nature* 282, 39 (1979); Kingsman et al., *Gene* 7, 141 (1979); Tschemper, et al., *Gene* 10, 157 (1980)] is commonly used. This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 [Jones, *Genetics* 85, 12 (1977)]. The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Any plasmid vector containing a yeast-compatible promoter, origin of replication and

-47-

termination sequence is suitable for use in yeast. Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase [Hitzeman, et al., *J.Biol.Chem.* 255, 2073 (1980)] or other glycolytic enzymes [Hess, et al., *J.Adv.Enzyme Reg.* 7, 149 (1968); Holland et al., *Biochemistry* 17, 4900 (1978)].

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication [Fiers, et al., *Nature* 273, 113 (1978)].

For example, pSV2neo contains a gene for ampicillin resistance neomycin resistance, which is under the control of an SV40 promoter. Thus, pSV2neo provides easy means for identifying cells transformed with genes for both the animal variable region and human constant region.

Preparation of Chimeric DNA

The genes coding for the heavy chain or the light chain will be constructed by joining the 5'-end of a DNA fragment which encodes the constant region to the 3' end of a DNA fragment which encodes the variable region. The DNA sequence coding for the antibody amino acid sequence may be obtained in association with the promoter and replication site from genomic DNA. To the extent that the host cells recognize the transcriptional regulatory and translational initiation signals

-48-

associated with the heterologous genes, then the region 5' and 3' of the variable region coding sequence may be retained with the variable region coding sequence and employed for transcriptional and translational initiation regulation. The non-coding region 3' to the constant region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. In referring to 5' or 3' for a double strand, it is intended to mean the direction of transcription, with 5' being upstream from 3'.

The intron sequence between the variable region for each respective chain may be joined to the corresponding human constant DNA fragment at any convenient restriction site. In providing a fragment encoding the variable region, it will usually be desirable to include a portion of the intron downstream from the J region. Where the intron is retained, it will be necessary that there be functional splice acceptor and donor sequences at the intron termini. The contiguous non-coding region 5' to the variable region will normally include those sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence and CAAT sequence. Usually, the 5'-non-coding sequence does not exceed about 1-2 kilo bases (kb).

An enhancer sequence should exist between the J region and the constant region. The enhancer employed may be the enhancer of either (1) the animal V region or the (2) the human constant region.

By retaining the 3'-region naturally contiguous to the DNA sequence coding for the constant region, the transcriptional termination signals may be provided for

the gene. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted. Conveniently, the non-coding 3' region may be obtained from a non-coding contiguous 3' region of a constant region from the expression host. The 3'-non-coding region may be joined to the constant region by any of the means described previously for manipulation and ligation of DNA fragments. This region could then be used as a building block in preparing the gene.

Preparation of Expression Vehicles

Construction of suitable expression vehicles containing the desired coding and control sequences may be produced as follows. The termini of the vectors and DNA fragments may then be religated to form the desired expression vehicles. The methods employed are not dependent on the DNA source, or intended host.

DNA fragments coding for the light chain and heavy chain may be inserted into separate expression vehicle, or into the same vector. Preferably, the fused genes encoding the light and heavy chimeric chains are assembled in two different expression vectors which can be used to cotransform a recipient cell, either concurrently or sequentially.

The means for insertion of the DNA fragments containing the chimeric genes into expression vectors includes using restriction endonucleases. "Restriction endonucleases" (or "restriction enzymes") are hydrolytic enzymes capable of catalyzing site-specific cleavage of DNA molecules. The locus of restriction endonuclease

-50-

action is determined by the existence of a specific nucleotide sequence. Such a sequence is termed the recognition site for the restriction endonuclease. Many restriction endonucleases from a variety of bacterial species have been isolated and characterized in terms of the nucleotide sequence of their recognition sites. Some restriction endonucleases hydrolyze the phosphodiester bonds on both strands at the same point, producing blunt ends. Others catalyze hydrolysis of bonds separated by a few nucleotides from each other, producing free single stranded regions at each end of the cleaved molecule. Such single stranded ends are self-complementary, hence cohesive, and may be used to rejoin the hydrolyzed DNA. Exemplary restriction enzymes include *Aat* II, *Bam* HI, *Eco* RI, *Hind* III, *Nde* I, *Spe* I, *Xba* I, *Sac* I, *Bgl* II, *Pst* I, *Sal* I and *Pvu* II.

Additionally, the expression vector may have a polylinker inserted therein which has a plurality of unique restriction sites. By digestion of the expression vector with the appropriate restriction enzymes, the polylinker will be cleaved so that at least one DNA fragment containing the gene can be inserted. Where the polylinker allows for distinguishable termini, the DNA fragment can be inserted in a single orientation; were the termini are the same, insertion of the DNA fragment will result in plasmids having two different orientations.

Cleavage is performed by treating the plasmid with a restriction enzyme(s). In general, about 10 µg plasmid or DNA fragments is used with about 10 units of enzyme in about 100 µl of buffer solution. Endonuclease digestion will normally be carried out at temperatures ranging from 37° to 65°C, at a pH of from 7 to 9.

-51-

(Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturers.) Time for the reaction will be from 1 to 18 hours.

5 It may be useful to prevent religation of the cleaved vector by pretreatment with alkaline phosphatase. Specific conditions are prescribed by the manufacturer.

10 After the restriction enzyme digest is complete, protein is removed by extraction with phenol and chloroform. The nucleic acid is recovered from the aqueous fraction (containing about 0.3M sodium acetate) by precipitation with about 2.5 volumes of ethanol.

15 Descriptions of methods of cleavage with restriction enzymes may be found in the following articles: Greene et al., Methods in Molecular Biology, Vol. 9, ed. Wickner, R. B., Marcel Dekker, Inc.,
20 New York, "DNA Replication and Biosynthesis"; Mertz and Davis, *Proc.Nat.Acad.Sci.,(USA)*, 69, 3370 (1972).

25 Size separation of the cleaved fragments by agarose gel electrophoresis is readily performed to follow the course of the reaction. Once the digestion has gone to the desired degree, the endonuclease may be inactivated by heating above 65°C for about 10 minutes or organic extraction.

30 The desired fragment is then purified from the digest. Suitable purification techniques include gel electrophoresis or sucrose gradient centrifugation.

-52-

The plasmid vehicle and foreign DNA fragments are then ligated with DNA ligase to recircularize. This process is referred to as annealing and DNA ligation.

5 An appropriately buffered medium containing the DNA fragments, DNA ligase, and appropriate cofactors is employed. The temperature employed will be between 25°
10 to 4°C. When DNA segments hydrogen bond, the DNA ligase will be able to introduce a covalent bond between the two segments. The time employed for the annealing will vary with the temperature employed, the nature of the salt solution, as well as the nature of the sticky ends or cohesive termini. Generally, the time for ligation may be from 5 to 18 hours. See Maniatis T., *Molecular*
15 *Cloning*, Cold Spring Harbor, *supra*.

Host Cells

Thereafter, the expression vehicle constructs may be used to transform an appropriate host cell.
20 Suitable host cells include cells derived from unicellular as well as multicellular organisms.

The chimeric immunoglobulin genes can be expressed in nonlymphoid cells such as bacteria or
25 yeast.

Various unicellular microorganisms can be transformed, such as bacteria. That is, those unicellular organisms which are capable of being grown
30 in cultures or fermentation. Since bacteria are generally the most convenient organisms to work with, bacteria will be hereinafter referred to as exemplary of the other unicellular organisms. Bacteria, which are susceptible to transformation, include members of the Enterobacteriaceae, such as strains of *Escherichia coli*;

-53-

Salmonella; Bacillaceae, such as *Bacillus subtilis*;
Pneumococcus; *Streptococcus*, and *Haemophilus influenzae*.

When expressed in bacteria, the immunoglobulin heavy chains and light chains become part of inclusion
5 bodies. The chains then must be isolated, purified and then assembled into functional immunoglobulin molecules.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used.
10 *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. The presence of the *trp1* lesion as a characteristic of the yeast host cell genome provides an effective environment
15 for detecting transformation by growth in the absence of tryptophan.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be
20 used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture, provided that the cell line is one that at least originally produced antibodies. Propagation of
25 vertebrate cells in culture has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are Sp2/0, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK,
30 COS-7 and MDCK cell lines.

The preferred recipient cell line is a plasmacytoma cell such as B lymphocytes or hybridoma cells. Plasmacytoma cells can synthesize, assemble and secrete immunoglobulins encoded by transformed

immunoglobulin genes. Further, they possess the mechanism for glycosylation of the immunoglobulin. Sp2/0 is a preferred recipient cell because it is an immunoglobulin-nonproducing plasmacytoma cell. The cell produces only immunoglobulin encoded by the transformed immunoglobulin genes. Plasmacytoma cells can be grown in culture or in the peritoneum of mice where secreted immunoglobulin can be obtained from ascites fluid.

Transformation of Host Cells

Transformation of host cells is accomplished as follows. The expression vehicle is linearized and the DNA is inserted into host cells for production of the antibody. Exemplary methods for inserting the DNA into host cells include electroportion, protoplast fusion, calcium phosphate-precipitation, or other conventional techniques, which use dextran sulfate, and PEG.

If cells without formidable cell wall barriers are used as host cells, transformation may be carried out by the calcium phosphate precipitation method as described by Graham and Van der Eb, *Virology*, 52, 546 (1978).

If prokaryotic cells or cells which contain substantial cell wall constructions are used, the preferred method of transformation is calcium treatment using calcium chloride as described by Cohen, F.N. et al, *Proc.Natl.Acad.Sci.(USA)* 69, 2110 (1972).

The host cells may be transformed via either co-transformation or targeted transformation.

For co-transformation, the genes coding for the light chain and heavy chain may be used to transform

-55-

5 separate cell cultures, either of the same or of
differing species; separate plasmids for light and heavy
chain may be used to co-transform a single cell culture;
or finally, a single expression plasmid containing both
genes and capable of expressing the genes for both light
and heavy chain may be transformed into a single cell
culture.

10 In the targeted transformation technique, the
host cells are transformed with genes encoding for the
light chain, and the cells containing the light chain
marker are selected. The light chain is found using
cytostaining or possibly by detection of the light chain
in the supernatant if it has been secreted. Cells
15 selected to have the light chain are transformed with
the heavy chain construct, and resultant cells
additionally containing the heavy chain marker selected.

20 It is known that some immortalized lymphoid
cell lines, such as plasmacytoma cell lines, in their
normal state secrete isolated Ig light or heavy chains.
Consequently, if such a cell line is transformed with
the vector containing the chimeric heavy or light chain
of the present invention, it will not be necessary to
25 transform the cell line or another cell line with the
other Ig chain, provided that the normally secreted
chain is complementary to the variable domain of the Ig
chain encoded by the vector initially used to transform
the cell line.

30

Selection and Expression of Transformed Host Cells

Generally, after transformation of the host
cells, the cells may be grown for about 48 hours to
allow for expression of marker genes. The cells are

-56-

then placed in a selective medium, where untransformed cells are killed, leaving only cells transformed with the DNA constructions.

5 Heavy and light chains or portions thereof, may be produced in isolation from each other and antibodies and fragments thereof may be obtained. Such preparations require the use of techniques to reassemble isolated chains.

10 The ability of the method of the invention to produce heavy and light chains or portions thereof, in isolation from each other offers the opportunity to obtain unique assemblies of immunoglobulins, Fab regions, and univalent antibodies. It is possible to
15 recombine the heavy and light chains *in vitro*, disrupted by cleavage of only the interchain disulfides, and to regain antibody activity even without restoration of the inter-chain disulfides [see Edelman, G.M., et al., *Proc. Natl. Acad. Sci. (USA)* 50, 753 (1963)].
20

 The transformed cells are grown under conditions appropriate to the production of the light chains and/or heavy chains, and assayed for heavy and/or
25 light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

30 The binding affinity of monoclonal antibodies for TAG72 is determined by means well known in the art (see Heyman, B. et al., *J. Immunol. Methods* 68, 193-204 (1984) and as described in detail in the Examples provided hereinafter).

-57-

Selected positive cultures are subcloned in order to isolate pure transformed colonies. A suitable technique for obtaining subclones is via the limited dilution method taught by McKeara in *Monoclonal Antibodies*, Plenum Press, N.Y. (1980).

5

Hybridomas that produce such chimeric antibodies may be grown using known procedures. The transformed cells can secrete large quantities of the light chains and/or heavy chains by culture *in vitro*, such as by hollow fiber systems, spinner culture, static culture, or *in vivo* such as ascites production.

10

The chimeric antibodies may be produced in large quantities by injecting a hybridoma into the peritoneal cavity of pristane-primed mice, and after an appropriate time (about 1-2 weeks), harvesting ascites fluid from the mice, which yields a very high titer of homogeneous monoclonal antibody, and isolating the monoclonal antibodies therefrom by methods well known in the art [see Stramignoni, P. et al., *Intl. J. Cancer* 31, 543-552 (1983)]. The hybridomas are grown up *in vivo*, as tumors in animals, the serum or ascites fluid of which can provide up to about 50 mg/mL of monoclonal antibodies. Usually, injection (preferably intraperitoneal) of about 10^6 to 10^7 histocompatible hybridoma cells into mice or rats will result in tumor formation after a few weeks. The antibodies can then be collected and processed by well known methods. (See generally, Immunological Methods, vols. I & II, eds. Lefkovits, I. and Pernis, B., (1979 & 1981) Academic Press, New York, N.Y.; and Handbook of Experimental Immunology, ed. Weir, D., (1978) Blackwell Scientific Publications, St. Louis, MO.)

15

20

25

30

-58-

The antibodies can then be stored in various buffer solutions such as phosphate buffered saline (PBS), which gives a generally stable antibody solution for further use.

5 The chimeric antibodies of the present invention may be fragmented using known protease enzymes, for example papain and pepsin, to obtain highly immunoreactive F(ab')₂, F(ab') and Fab fragments. In addition, active fragments of Ig formed by proteolysis
10 (approximately 50,000 MW) can be split into their fully reduced heavy chain and light chain components and fairly efficiently reconstructed to give an active antibody [Haber, E., *Proc.Natl.Acad.Sci.(USA)* 52, 1099
15 (1964); Whitney, P.L., et al., *Proc.Natl.Acad.Sci.(USA)* 53, 524 (1965)]. The reactivity of the resulting F(ab')₂, F(ab') and Fab fragments are determined by methods as described above for the complete monoclonal antibody molecule.

20

25

30

Uses of the Antibodies

5 The antibodies of the present invention, as
well as immunoreactive fragments or recombinants
thereof, provide unique benefits for use in a variety of
cancer treatments. In addition to the ability to bind
specifically to malignant cells and to localize tumors,
the antibodies have constant variable regions which do
not bind detectably to normal cells such as fibroblasts,
10 endothelial cells, or epithelial cells in the major
organs.

Specifically, the antibodies, immunoreactive
fragments or recombinants thereof are useful for, but
15 not limited to, the following types of cancer treatment:
(1) *in vivo* diagnostic assays conjugated to an imaging
marker, for the *in situ* detection of carcinoma lesions, as
further described below; (2) *in vivo* therapy, using the
antibodies of the present invention alone or conjugated
20 to a therapeutic agent such as a radionuclide, toxin,
effector cells, other antibodies or via a complement
mechanism, as described below; and (3) radioimmunoguided
surgery, as described below.

25 Moreover, a pharmaceutical composition
comprising the antibodies of the present invention in a
pharmaceutically acceptable, non-toxic, sterile carrier
such as physiological saline, non-toxic buffers and the
like, is also now possible.
30

Injectable compositions of the present
invention may be either in suspension or solution form.
In solution form the complex (or when desired the
separate components) is dissolved in a pharmaceutically
acceptable carrier. Such carriers comprise a suitable

-60-

5 solvent, preservatives such as benzyl alcohol, if needed, and buffers. Useful solvents include, for example, water, aqueous alcohols, glycols, and phosphonate or carbonate esters. Such aqueous solutions contain no more than 50% of the organic solvent by volume.

10 Injectable suspensions as compositions of the present invention require a liquid suspending medium, with or without adjuvants, as a carrier. The suspending medium can be, for example, aqueous polyvinylpyrrolidone, inert oils such as vegetable oils or highly refined mineral oils, or aqueous carboxymethylcellulose. Suitable physiologically acceptable adjuvants, if
15 necessary to keep the complex in suspension, may be chosen from among thickeners such as carboxymethylcellulose, polyvinylpyrrolidone, gelatin, and the alginates. Many surfactants are also useful as suspending agents, for example, lecithin, alkylphenol,
20 polyethylene oxide adducts, naphthalenesulfonates, alkylbenzenesulfonates, and the polyoxyethylene sorbitan esters. Many substances which effect the hydrophobicity, density, and surface tension of the liquid suspension medium can assist in making injectable
25 suspensions in individual cases. For example, silicone antifoams, sorbitol, and sugars are all useful suspending agents.

30 Because cancer cells are heterogeneous and consequently, a single monospecific chimeric antibody may not be able to recognize all cells expressing different epitopes of a tumor.

 Thus, it may be desirable to administer several different chimeric antibodies of the present invention.

-61-

The sequential use of these various antibodies should substantially reduce the anti-idiotypic responses in human patients when compared to repeated use of a single antibody. For example, CH92, CH88, and CH44 could be sequentially administered to a patient. Since these
5 antibodies have different light chains and, in fact different CDR3 regions anti-idiotypic responses should be minimized.

In Vivo Diagnostic Assays

In vivo diagnostic assays of human tumors or metastasis thereof using the antibodies, immunoreactive fragments or recombinants thereof are conjugated to a marker, administered to a patient, and then the presence
15 of the imaging marker in the patient is detected by exposing the patient to an appropriate detection means.

Administration and detection of the antibody-
-imaging marker conjugate as well as methods of
20 conjugation of the antibody to the imaging marker are accomplished by methods readily known or readily determined, as described, for example, in Goldenberg, D.M. et al., *New England J. Med.*, 298, 1384-1388 (1978);
25 Goldenberg, D.M. et al., *J. Amer. Med. Assoc.* 280, 630-635 (1983); Goldenberg, D.M. et al., *Gastroenterol.* 84, 524-532 (1983); Siccardi, A.G. et al., *Cancer Res.* 46, 4817-4822 (1986); Epenetos, A.A. et al., *Cancer* 55, 984-987 (1985); Philben, V.J. et al., *Cancer* 57, 571-576 (1986);
30 Chiou, R. et al., *Cancer Inst.* 76, 849-855 (1986); Colcher, E. et al., *Cancer Res.*, 43, 736-742 (1983); Colcher, E. et al., Laboratory Research Methods in Biology and Medicine Immunodiagnosics, New York, Alan R. Liss, pp. 215-258 (1983); Keenan, A.M. et al., *J. Nucl. Med.* 25, 1197-1203 (1984); Colcher D. et al., *Cancer Res.* 47, 1185-1189

(1987); Estaban, J.M. et al., *Intl.J.Cancer* 39, 50-59
(1987); Martin, D.T., et al., *Curr.Surg.* 41, 193-194
(1984); Martin, E.W. Jr. et al., *Hybridoma* 5, S97-S108
(1986); Martin, D.T. et al., *Am.J.Surg.* 150, 672-675
(1985); Meares et al., *Anal.Biochem.* 142, 68-78 (1984);
5 and Krejcarek et al., *Biochem.and Biophys.Res.Comm.* 77,
581-585 (1977).

The dosage will vary depending upon the age and
weight of the patient. Generally, the dosage should be
10 effective to visualize or detect tumor sites, distinct
from normal tissues. Preferably, a one-time dosage will
be between 0.1 to 200 mg of an antibody-marker conjugate
per patient.

15 Examples of imaging markers which can be
conjugated to the antibody are well known to those
skilled in the art and include substances which can be
detected by diagnostic imaging using a gamma scanner or
20 hand held gamma probe or Positron Emission Tomography or
the like, as described in the references cited above,
and substances which can be detected by nuclear magnetic
resonance imaging using a nuclear magnetic resonance
spectrometer or the like, as described in the references
25 cited above.

Suitable examples of substances which can be
detected using a gamma scanner or the like include, for
example, radioisotopes such as ^{125}I , ^{131}I , ^{123}I , ^{111}In ,
30 ^{105}Rh , ^{153}Sm , ^{67}Cu , ^{67}Ga , ^{166}Ho , ^{177}Lu , ^{186}Re , ^{188}Re ,
and $^{99\text{m}}\text{Tc}$. ^{125}I , ^{123}I , ^{153}Sm and $^{99\text{m}}\text{Tc}$ are preferred
due to their low energy and suitability for long range
detection.

An example of a substance which can be detected using a nuclear magnetic resonance spectrometer or the like is gadolinium (Gd).

In Vivo Cancer Treatment

5 In this method, the antibody-therapeutic agent conjugate can be delivered to the carcinoma site thereby directly exposing the carcinoma tissue to the therapeutic agent.

10 The antibodies of the present invention, immunoreactive fragments or recombinants thereof may be administered in a pharmaceutically effective amount for the *in vivo* treatment of human carcinomas or metastasis
15 thereof. A "pharmaceutically effective amount" of the antibody, immunoreactive fragment or recombinant thereof, conjugated or unconjugated to a therapeutic agent, means the amount of said antibodies in the pharmaceutical composition should be sufficient to
20 achieve effective binding with the antigens against which said antibodies have specific affinity. The pharmaceutical composition may be administered in a single or multiple dosage.

25 Methods of preparing and administering conjugates of the antibody, immunoreactive fragments or recombinants thereof and a therapeutic agent are well known or readily determined by those skilled in the art.
30 Moreover, suitable dosages will depend on the age and weight of the patient and the therapeutic agent employed and are well known or readily determined by those skilled in the art. Representative protocols are described in the references cited below.

Examples of the antibody-therapeutic agent conjugates which can be used in therapy include the following: (1) antibodies coupled to radionuclides, such as ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , ^{211}At , ^{67}Ga , ^{125}I , ^{186}Re , ^{188}Re , ^{177}Lu , $^{99\text{m}}\text{Tc}$, ^{153}Sm , ^{123}I and ^{111}In as described, for example, in Goldenberg, D.M. et al., *Cancer Res.* 41, 4354-4360 (1981); Carrasquillo, J.A. et al., *Cancer Treat. Rep.* 68, 317-328 (1984); Zalcbberg, J.R. et al., *J. Natl. Cancer Inst.* 72, 697-704 (1984); Jones, D.H. et al., *Int. J. Cancer* 35, 715-720 (1985); Lange, P.H. et al., *Surgery* 98, 143-150 (1985); Kaltovich, F.A. et al., *J. Nucl. Med.* 27, 897 (1986), Order, S.E. et al., *Int. J. Radiother. Oncol. Biol. Phys.* 8, 259-261 (1982), Courtenay-Luck, N. et al., *Lancet* 1, 1441-1443 (1984) and Ettinger, D.S. et al., *Cancer Treat. Rep.* 66, 289-297 (1982); (2) antibodies coupled to drugs or biological response modifiers such as methotrexate, adriamycin, and lymphokines such as interferon as described, for example, in Chabner, B. et al., Cancer, Principles and Practice of Oncology, Philadelphia, PA, J.B. Lippincott Co. Vol. 1, pp. 290-328 (1985); Oldham, R.K. et al., Cancer, Principles and Practice of Oncology, Philadelphia, PA, J.B. Lippincott Co., Vol. 2, pp. 2223-2245 (1985); Deguchi, T. et al., *Cancer Res.* 46, 3751-3755 (1986); Deguchi, T. et al., *Fed. Proc.* 44, 1684 (1985); Embleton, M.J. et al., *Br. J. Cancer* 49, 559-565 (1984) and Pimm, M.V. et al., *Cancer Immunol. Immunother.* 12, 125-134 (1982); (3) antibodies coupled to toxins, as described, for example, in Uhr, J.W. et al., Monoclonal Antibodies and Cancer, Academic Press, Inc., pp. 85-98 (1983), Vitetta, E.S. et al., Biotechnology and Bio. Frontiers, Ed. P.H. Abelson, pp. 73-85 (1984) and Vitetta, E.S. et al., *Sci.*, 219, 644-650 (1983); (4) heterofunctional antibodies, for example, antibodies

coupled or combined with another antibody so that the complex binds both to the carcinoma and effector cells, e.g., killer cells such as T cells, as described, for example, in Perez, P. et al., *J. Exper. Med.* 163, 166-178 (1986); and Lau, M.A. et al. *Proc. Natl. Acad. Sci. (USA)* 82, 8648-8652 (1985); and (5) native, i.e., non-conjugated or non-complexed, antibodies, as described in, for example, Herlyn, D. et al., *Proc. Natl. Acad. Sci., (USA)* 79, 4761-4765 (1982); Schulz, G. et al., *Proc. Natl. Acad. Sci., (USA)* 80, 5407-5411 (1983); Capone, P.M. et al., *Proc. Natl. Acad. Sci., (USA)* 80, 7328-7332 (1983); Sears, H.F. et al., *Cancer Res.* 45, 5910-5913 (1985); Nepom. G.T. et al., *Proc. Natl. Acad. Sci., (USA)* 81, 2864-2867 (1984); Koprowski, H. et al., *Proc., Natl. Acad. Sci., (USA)* 81, 216-219 (1984); and Houghton, A.N. et al., *Proc. Natl. Acad. Sci., (USA)* 82, 1242-1246 (1985).

The methods for combining the antibody or antibody fragment to a desired therapeutic agent as described above are conventional and well known in the art. For example, the methods given in the references above.

RadioImmunoguided Surgery

Antibodies, immunoreactive fragments or recombinants thereof, are important for radioimmunoguided surgery (RIGS). In RIGS, an intraoperative therapy, tumors are localized and excised. An antibody labeled with an imaging marker is injected into the patient, and bound antibody localized by a hand-held gamma detecting probe (GDP) and excised. An exemplary GDP is Neoprobe™, commercially available from Neoprobe™ Corporation, Tampa, FL. See Martin et al., "Radioimmunoguided surgery: a new approach to the

-66-

intraoperative detection of tumor using antibody B72.3",
Amer.J.Surg. 156, 386-392 (1988); Martin et al.

"Radioimmunoguided surgery: intraoperative use of
antibody 17-1A in colorectal cancer", *Hybridoma* 5, S97-
S108 (1986).

5

Administration and detection of the antibody-
-imaging marker conjugate as well as methods of
conjugation of the antibody to the imaging marker are
accomplished by methods readily known or readily
determined by one skilled in the art, as described, for
example, above.

10

15

The dosage will vary depending upon the age and
weight of the patient, but generally a one time dosage
of 0.1 to 200 mg of antibody-marker conjugate per
patient is sufficient.

20

25

30

The following nonlimiting examples are merely for illustration of the construction and expression of chimeric DNA sequences encoding the antibodies of this invention. All temperatures not otherwise indicated are in Centigrade. All percents not otherwise indicated are by weight.

Examples

10 Replacement of Mouse Constant Regions

CC antibodies were derived from mice, and are significantly less capable of carrying out the effector functions possessed by the human constant regions.

15 Consequently, in the following examples, selected antibodies are "humanized" by genetically removing the constant regions of the heavy and light chains and replacing them with their human equivalents.

20 The mouse light chain constant region genes were replaced with the human kappa (k) gene, and the mouse heavy chain genes were replaced with each of the four human gamma isotypes ($\gamma 1$, $\gamma 2$, $\gamma 3$ and $\gamma 4$). Each of
25 these four gamma isotypes possess unique biological properties. For a general review, see "The Human IgG subclasses", Hamilton, R.G. (1989) Doc. No. CB0051-289, Calbiochem Corporation.

30 Preparation of Heavy and Light Chain Variable Region

Isolation of CC49 light chain

CC49 hybridoma cells secrete an antibody having an IgG₁ isotype heavy chain and a kappa light chain.

-68-

Total DNA from CC49 hybridoma cells, Balb/C mouse kidney cells and NSI plasmacytoma cells was isolated according to the procedures set forth in *Cell*; 24, 353-356 (1981).

5 Generally, about 10-20 µg of the extracted DNA from each cell line was digested to completion with 80 units of *Bam* HI, *Eco* RI, *Hind* III, *Spe* I, *Xba* I, *Sac* I, *Bgl* II, and *Pst* I in 50-100 microliters of a reaction mixture containing the appropriate reaction buffer at
10 37°C overnight.

 Next, the total extracted DNA from each cell line was subjected to the Southern hybridization technique, developed by E. M. Southern, [*J.Mol.Biol.* 98, 503-517 (1975)]. The DNA fragments were fractionated on
15 the basis of their size by means of electrophoresis on a 0.8% agarose gel. The double-stranded DNA fragments were modified into single-stranded DNA fragments in an
20 alkali solution; and then a nitrocellulose filter was placed into close contact with the gel to transfer the modified DNA segments onto the filter in the presence of a high salt concentration solution.

25 Hybridization was carried out using, as the probe, a random primed <32P>-labelled L chain.

 More specifically, the probe was a 1.71 kilo base pair (kbp) *Hind* III-*Pst* I fragment containing the
30 coding exons for the murine J_L regions (J1-J5) and was isolated from the plasmid pGD1. A nucleotide sequence of the probe fragment is provided in Figure 7. This plasmid is described in "Site Directed Cleavage of Immunoglobulin Gene Segments by Lymphoid Cell Extracts", Agostaro et al., *Can.J.Biochem.Cell Biol.* 63, 969-976

-69-

(1985). The plasmid was provided by Nobumichi Hozumi and John Roder, Mt. Sinai Research Institute, Toronto, Ontario, Canada.

5 To radiolabel the probe, alpha<32P> dCTP was obtained from Amersham, Arlington Heights, IL, USA, and the random priming kit was obtained from Pharmacia, Piscataway, NJ, USA.

10 The signals in Southern transfers were visualized by autoradiography using Kodak X-OMAT™ AR film. No obviously rearranged band was observed. Thus, relative to the standards, no unique band was detected on the autoradiogram for the CC49 DNA digested with *Hind*
15 III. It could not be ruled out from the Southern data, however, that the rearranged band for the L chain was masked by a band migrating in the CC49 *Hind* III digested DNA parallel to the band resulting from a *Hind* III
20 digest of mouse kidney cell DNA (representing the germline DNA). This actually turned out to be the case.

Preparation of Plasmid Containing Mouse V_L Genes

25 LAMBDA-ZAP™, a lambda-based insertion cloning vector capable of self excision, was purchased from Stratagene Co., La Jolla, CA, USA. LAMBDA-ZAP™ is described on pages 20-21 of the 1987 Stratagene catalog. The cohesive (cos) ends of LAMBDA-ZAP™ were ligated overnight by following the manufacturer's protocol.

30 Twenty micrograms of the ligated LAMBDA-ZAP™ were digested with 5 microliters (15 units) of *Spe* I, purchased from New England Biolabs, Inc. The total volume of the digest was 100 microliters. After 55 minutes of digestion, another 6 units of *Spe* I were

-70-

added. After 70 minutes, the reaction was stopped by phenol extraction and ethanol precipitation carried out as per Stratagene's protocol.

Digestion with *Spe* I restriction enzyme results in production of "sticky ends" at both termini. These sticky ends were modified with T4 DNA polymerase to create half filled-in *Spe* I sticky ends, e.g., 5'ACT/3'TCATG. To accomplish the half fill-in reaction, the DNA pellet obtained in the ethanol precipitation above was dissolved in 8 microliters of water. To this was added 2 microliters of 10 millimolar dTTP, 2 microliters of 10 millimolar dCTP, 2 microliters of Stratagene's 10X ligase buffer, 4 microliters of reionized, distilled water, and 2 microliters of a Klenow fragment from Bethesda Research Laboratories (BRL). The reaction was carried out at ambient temperatures for 30 minutes. The reaction was stopped by inactivating the DNA polymerase at 65°C for 10 minutes.

One hundred sixty micrograms of total CC49 hybridoma DNA (containing the mouse light chain promoter and the L and VJ exons) were digested to completion with *Hind* III. Fragments between about 1 kb to about 20 kb were cut out of 0.8% agarose gels. The DNA was purified using GENECLAN™, which is commercially available from BIO 101 (La Jolla, CA, USA).

The total CC49 hybridoma DNA *Hind* III digested fragments were half-filled similarly to the LAMBDA-ZAP™'s *Spe* I fragments with the exception that dATP and dGTP were employed. The half-filled *Hind* III digested fragments produced 5'AGCTT/3'GAA sticky ends, which are

-71-

compatible with the *Spe* I half-filled λ LAMBDA-ZAP™ fragment above.

After phenol extraction and ethanol precipitation, according to the teachings of *Maniatis*,
5 the total CC49 hybridoma *Hind* III modified and LAMBDA-ZAP™ *Spe* I modified DNA fragments were ligated by means of T4 DNA ligase. The ligation reaction was set using a 6.1 microliter ligation mixture containing the
10 following: about 0.2 micrograms of the total CC49 hybridoma *Hind* III modified DNA in a 3 microliter solution, about 1 microgram of LAMBDA-ZAP™ *Spe* I modified DNA in a 1 microliter solution, 0.6 microliters of Stratagene's 10X ligase buffer, 0.5 microliters 10 millimolar ATP, and 1 microliter of Stratagene ligase.
15 This was incubated overnight in a water bath and the temperature lowered incrementally from about 18°C to about 4°C. This ligation eliminated both the *Hind* III and the *Spe* I sites.

20 A genomic library of ligated mix was made according to Stratagene's protocol. Briefly, 2 microliters of the ligation mix produced above was used in Stratagene's Gigapack Gold packaging system,
25 following the directions of the manufacturer. Fifteen 150 mm plates having a density of 50,000 plaques per plate were screened, as per manufacturer's directions, for positive clones by hybridization to nitrocellulose filters, obtained from Schleicher-Schuell, Keene, NH,
30 USA. The <32P> random-labelled probe derived from pGD1, which was described above, was used for hybridization. Two positive clones were obtained.

Each clone was plaque purified and recombinant plasmids (phagemids) of LAMBDA-ZAP™ containing the CC49

-72-

L chain variable region were obtained by using Stratagene's automatic excision protocol. The vector portion of the resulting recombinant plasmid is called pBLUESCRIPT SK(-) and consists of 2964 bp as described in the 1987 Stratagene catalog. A plasmid map of
5 pBLUESCRIPT SK(-) is shown in Fig. 8.

The DNA from the two positive clones was partially sequenced and both were identical. One of the clones, which was named pRL101, was used for further
10 studies.

Restriction Mapping of CC49 Light Chain

pRL101 was 7.61 kb, and the size of the DNA
15 insert was determined by restriction enzyme mapping to be 4.65 kb. A plasmid map of pRL101 is shown in Figure 9. A restriction enzyme map of the CC49 L chain genomic DNA insert in pRL101 is shown in Fig. 10.

20 Isolation of CC83 Light Chain Variable Region

The procedures used to isolate the CC83 light chain were essentially those used to isolate the CC49 light chain, with the following exception.

25 A genomic library containing 7×10^5 plaques was screened using as the probe the $<^{32}\text{P}>$ random-labelled 1.71 *Hind* III-*Pst* I fragment derived from pGD1, as described above. One positive clone was obtained.
30 The positive clone was named pRL200.

Restriction Mapping of CC83 Light Chain

pRL200 was 7.44 kb, and the size of the DNA insert was determined by restriction enzyme mapping to be 4.48 kb. A plasmid map of pRL200 is shown in Figure

-73-

11. A restriction enzyme map of the CC83 L chain genomic DNA insert in pRL200 is shown in Fig. 12.

Isolation of CC49 Heavy Chain Variable Region

5 The procedures used to isolate the CC49 heavy chain were essentially those used to isolate CC49 light chain, including the screening of the same CC49 *Hind* III modified DNA.

10 The hybridization probe used to screen the library was generated from pNP9, which contains a 1.98 kbp *Eco* RI-*Bam* HI fragment containing the coding exons for JH3 and JH4 of the CC49 immunoglobulin heavy chain. The nucleotide sequence of the probe fragment is
15 provided in Figure 13. The plasmid was provided by Dr. Nobumichi Hozumi and Dr. John Roder, Mt. Sinai Research Institute, Toronto, Ontario, Canada.

 A genomic library containing 9.5×10^5 plaques
20 was screened, from which one positive clone was obtained. The positive clone was named pHH49.

Restriction Mapping of CC49 Heavy Chain

25 pHH49 was about 7.0 kb, and the size of the DNA insert was determined by restriction enzyme mapping to be about 4.0 kb. A plasmid map of pHH49 is shown in Figure 14.

Isolation of CC83 Heavy Chain Variable Region

30 The procedures used to isolate the CC83 heavy chain were essentially those used to isolated CC49 heavy chain, with the following exceptions.

-74-

About thirteen micrograms of ligated LAMBDA-ZAP™ vector DNA were digested with 12 units of *Spe* I, purchased from New England Biolabs, Inc., in a total of 100 microliters of an appropriate buffer. The LAMBDA-ZAP™ was digested at 37°C for one hour. The reaction
5 mixture was phenol extracted and ethanol precipitated as per Stratagene's protocol. The *Spe* I digested LAMBDA-ZAP™ was dephosphorylated according to procedures set forth in *Maniatis* except that 40 fold excess of calf
10 intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA) was used.

DNA from CC83 was digested to completion with *Spe* I. Fragments between about 3 kb to about 40 kb were
15 isolated from a 0.8 percent agarose gel slice by electroelution as described by *Maniatis*, and ligated with the dephosphorylated *Spe* I-cut LAMBDA-ZAP™ vector.

A genomic library containing 5×10^5 plaques
20 was screened using the probe generated from pNP9, the sequence of which is provided in Figure 13. One positive clone was obtained. The positive clone was named pHS83.

25 Restriction Mapping of CC83 Heavy Chain

pHS83 was 7.95 kb, and the size of the DNA insert was determined by restriction enzyme mapping to be about 5 kb. A plasmid map of pHS83 is shown in
30 Figure 15.

Sequencing of CC46, CC49, CC83 and CC92 mRNA

Total RNA from about 1×10^7 CC49 cells frozen at -70°C was extracted essentially as reported by *Maniatis*, with the following exceptions. Four molar

-75-

guanidinium isothiocyanate and 2.5 molar sodium citrate, pH 7.0, and a SW40Ti rotor centrifuged at 31,000 rpm were used.

A total of 2.7 mg of CC49 RNA was isolated.

- 5 After centrifugation, poly A+ mRNA was purified from about 1.68 mg of RNA by oligo(dT)-cellulose chromatography using Type 3 oligo(dT)-cellulose obtained from Collaborative Research, Inc., Bedford, MA, USA. The procedure was as described by Aviv and Leder, *Proc. Nat'l. Acad. Sci.(USA)* 69, 1408 (1972). A total of 50.24 μ g of poly A+ mRNA was obtained from 1.68 milligrams of mRNA.

- 15 A total of 3.82 mg of CC83 RNA was isolated from approximately 1×10^7 cells. A total of 54.6 μ g of poly A+ mRNA was isolated from 1.91 milligrams of total RNA.

- 20 A total of 0.814 mg of CC92 RNA was isolated from approximately 2.6×10^8 cells. A total of 41.88 micrograms of poly A+ RNA was isolated from 0.814 mg of total RNA.

- 25 A total of 1.7 mg of CC46 RNA was isolated from approximately 2.89×10^8 cells. A total of 68.88 micrograms of poly A+ RNA was isolated from 1.7 mg of total RNA.

- 30 Synthetic oligonucleotide primers were synthesized using an Applied Biosystems' (Applied Biosystems (ABI), Foster City, CA, USA) Model 380A DNA synthesizer, by phosphoramadite-based chemistry as specified by ABI. The oligonucleotides were purified, as specified by the manufacturer, after electrophoresis on a 20% polyacrylamide gel containing 7M urea.

-76-

Oligonucleotide concentrations were determined spectrophotometrically at an optical density of 260 nm, where 1 OD 260 nm unit is equal to 33 µg/mL of single-stranded DNA.

5 The following oligonucleotide primers were made for mRNA sequencing: (1) For the CC49, CC83 and CC92 light chains, K_L(-), a 22-mer:

5'-GGAAGATGGATACAGTTGGTGC-3'

10 complimentary to the coding sequence of the 5' end of the constant region for mouse immunoglobulin kappa chains, is used to determine the 3' most mRNA sequence of the light chain variable region.

15 Additionally, for CC49 light chain, 49FR1(-), a 17-mer:

5'-GGAAGATGGATACAGTTGGTGC-3'

was used to determine the remaining sequence.

20 Additionally, for CC83 light chain, J4(-), a 24-mer:

5'-CCAACTTTGTCCCCGAGCCGAACG-3'

and also 83L CDR2(-), a 17-mer:

5'-CAGGGACTCCAGTGTGC-3'

25 was used to determine the remaining sequence.

 Additionally, for CC92 light chain, J5(-):

5'-CGTTTCAGCTCCAGCTTGGTCCC-3'

was used to determine the remaining sequence.

30 For the CC46, CC49, CC83, and CC92 γ1 heavy chains, CH1(-), a 24-mer:

5'-ATGGAGTTAGTTTGGGCAGCAGAT-3'

complimentary to the coding sequence of the 5' end of the murine γ1 heavy chain constant region. The CH1 (-)

-77-

24-mer is used to determine the 3'-most mRNA sequence of heavy chain variable regions.

Additionally, for the CC49 heavy chain, JH4(-)-20mer:

5 5'-GGTGACTGAGGTTCTTGAC-3'
was used to determine the remaining sequence.

Additionally, for the CC83 heavy chain, JH2(-)-16mer:

10 5'-CTGAGGAGACTGTGAG-3'
was used to determine the remaining sequence.

Additionally, for the CC92 heavy chain and the B72.3 heavy chain, B72.3/CC92 HC-20mer:

15 5'-CCTTGAAGTTCTCATTGTAC-3'
was used to determine the remaining sequence.

The following procedures were carried out as outlined by Jan Gelliebter in BRL *FOCUS* 2, 1 (1987).

20 The oligonucleotide primers were end-labelled as follows: 100 ng of oligonucleotide were combined in 50mM Tris HCl (pH 8), 10mM MgCl₂, 5mM dithiothreitol, and 1mM spermidine, 100 μ Ci (γ -³²P) ATP (Amersham, 5000
25 Ci/mMole) and 7 units of T4 polynucleotide kinase in a volume of 13 μ l. The reaction was allowed to proceed at 37°C for 30 minutes, then heated for 5 minutes at 65°C to inactivate the kinase, and then 7 μ l of water was added to make the concentration 5 ng/ μ l. The labelled primers
30 were stored at -20°C until needed.

Separate samples, each containing about 13 micrograms of poly(A)⁺ mRNA of CC49, CC83, CC92, or

-78-

CC46, respectively, were resuspended in 10 μ l of annealing buffer [10mM Tris HCl (pH 8.3), and 250mM KCl].

5 A 5 ng sample of end-labelled oligonucleotide primer was added to each mRNA sample, heated to 80°C for 3 minutes, and annealed for 45 minutes at 61°C, for the $K_L(-)$ and 65°C for the CH1(-) oligonucleotides. AMV reverse transcriptase (Boehringer Mannheim) was used at a level of 6 units for each mRNA sequencing reaction. The remainder of the sequencing was carried out as set
10 forth in BRL *FOCUS* 9, 1 (1987).

Initial sequence data showed that the heavy and light chains were rearranged as follows: CC49 kappa light chain used a J5; CC49 γ 1 heavy chain used a J_H4 .
15 The CC83 light chain used a J4; the CC83 gamma 1 used a J_H2 . The CC46 kappa light chain used a J2; the CC46 heavy chain used a J_H3 . The CC92 light chain used a J5; the CC92 gamma 1 used a J_H2 .

20 Figure 16 shows the nucleotide sequence of CC49 V_H , with the underlined segments showing the sequences derived using oligonucleotide primers on mRNA.

25 Figure 17 shows the nucleotide sequence of CC83 V_H , with the underlined segments show the sequences derived using oligonucleotide primers on mRNA.

30 The entire nucleotide sequences of CC46 V_H and CC92 V_H , shown in Figure 2, were derived using oligonucleotide primers on mRNA.

Figure 4a shows the nucleotide sequence of CC49 V_L , with the underlined segments show the sequences derived using oligonucleotide primers on mRNA.

-79-

Figure 5a shows the nucleotide sequence of CC83 V_L, with the underlined segments show the sequences derived using oligonucleotide primers on mRNA.

5 The entire nucleotide sequence of CC92 V_L, shown in Figure 6, was derived using oligonucleotide primers on mRNA.

Protein Sequence

10 Purified murine CC49 and CC83 immunoglobulin molecules were sent to Dr. George Tarr at the University of Michigan Protein Sequencing facility for NH₂-terminal amino acid sequence analysis. Dr. Tarr used the Edman degradation method, as modified by Tarr, G.E., in
15 "Manual Edman Sequencing System", Microcharacterization of Polypeptides: A Practical Manual [John E. Shively, ed., Humana Press, Inc., Clifton, N.J., pp 155-194 (1986)]. Briefly, Dr. Tarr reduced and alkylated the
20 the immunoglobulin molecules. The light and heavy chains of the immunoglobulin molecules were separated by reverse phase HPLC.

25 Figure 4b shows the amino acid sequence for CC49 V_L, with the results of the amino acid sequence determination for the first 24 amino acids of the mature CC49 V_L being underlined. Figure 5b shows the amino acid sequence for CC83 V_L, with the results of the amino acid sequence determination for the first 51 amino acids
30 of the mature CC83 V_L being underlined. ASN-20 could not be determined in the CC83 light chain, because of the presence of N-linked carbohydrate residues at this position, which is shown in the PNGase F experiment below. The sequence Asn-Ile-Thr corresponds to the

-80-

consensus sequence Asn-X-Thr/Ser for carbohydrate attachment to Asn.

Since the heavy chains of immunoglobulins CC49 and CC83 are blocked at the N-terminus and unavailable
5 for amino acid sequence determination, the native glycopeptide was treated with cyanogen bromide (CNBr) to cleave at the methionine residues. The cleavage resulted in fragments, which were purified by reverse
10 phase HPLC. N-terminal amino acid sequencing was performed on the CNBr fragments.

The results of the amino acid determination of one of the CC49 V_H CNBr peptide fragments are indicated as underlined residues in Figure 18. The results of the
15 amino acid determination of one of the CC83 V_H CNBr peptide fragments are indicated as underlined residues in Figure 19. As with CC49, all other peptide sequences correspond to CNBr fragments derived from the constant
20 region of mouse γ 1.

Determination of N-Linked Carbohydrate on CC83 L Chain

This experiment was done to verify that there is an N-linked carbohydrate attached to the CC83 light
25 chain, presumably at ASN-20 (see Figure 5b). The enzyme glycopeptidase F (PNGase F), which is isolated from the culture filtrate of *Flavobacterium meningosepticum* [Tarentino, A. L et al., *Biochemistry* 24, 4665-4671
30 (1985)], will cleave high mannose and/or biantennary complex sugars N-linked to ASN to generate a free carbohydrate structure and an ASP residue from the ASN to which it was attached. The difference in molecular weight between the glycosylated and unglycosylated form of the same peptide can be determined by SDS-PAGE.

Twelve microgram reactions with and without PNGase F (Boehringer Mannheim, Indianapolis, IN, USA) for the purified murine antibodies CC49, CC83 and CC11 F(ab')₂ (a positive control) were carried out in a final aqueous reaction volume of 40 microliters. Four
5 microliters of 10 x buffer (1M potassium phosphate, 0.1M disodium EDTA pH 7.4) were added to each reaction mix. To those tubes designated "with PNGase F", 7.5 microliters of PNGase F were also added and all tubes
10 were incubated at 37°C for 1 h. To the reaction tubes was added 40 microliters of Laemmli 2X sample dilution buffer containing β-mercaptoethanol. A 10 percent SDS polyacrylamide gel was electrophoresed, the gel stained with Coomassie Brilliant Blue R-250 and destained.
15 Figure 20 shows the results. As shown in lane 2, a new band (*) appears in the PNGase F treated CC83 sample but not in the untreated CC83 sample (lane 3). The new band is approximately 2,000-3,000 molecular weight smaller
20 than the native light chain band, which represents the removal of an N-linked carbohydrate moiety. The only consensus glycosylation site for the CC83 light chain is at ASN 20, so by inference it is assumed that this is the actual site of glycosylation and why it did not show
25 up on the N-terminal sequence analysis of the CC83 light chains as ASN. The CC49 light chain does not change mobility when treated with PNGase F (lane 6), but a new band is observed for the heavy chain fragment of CC11 F(ab')₂ (lane 4*) which serves as a positive control.
30 mRNA sequence data of CC11 heavy chain indicates a consensus glycosylation site in the V domain (data not shown). The standards (lane 1) are bovine serum albumin (BSA), MW 68,000 and soybean trypsin inhibitor (STI), MW 21,500.

DNA Sequence

Plasmid DNA was sequenced directly using the Sequenase DNA sequencing kit, obtained from United States Biochemical (USB), Cleveland, OH, USA. USB's
5 protocol was followed to sequence double stranded DNA. The DNA of each variable region was sequenced using the J_H or J_L oligo determined from the mRNA sequence information to be specific for each productively
10 rearranged heavy chain or light chain gene, respectively.

After the initial sequences were determined, the sequence was extended further by using additional primers. The additional primers were synthesized using
15 information gathered from the sequences previously generated.

Using the above technique, the DNA sequences of the entire heavy chain variable region exons and light
20 chain variable region exons of CC49 and CC83 were obtained. The DNA sequence was compiled and analyzed using Hitachi's DNA sequence analysis software program DNASIS™.

25 The following oligonucleotide primers were made for DNA sequencing:

- (1) For both light chains, C_K intron(-):
5'-GAAAACCTGTGTCTTACAC 3'.
- 30 (2) For the CC49 light chain, CC49 FRI(+):
5'-GTACCTGTGGGGACATTG 3',
and JK5(-)-23mer
5'-CGTTTCAGCTCCAGCTTGGTCCC-3'.
- (3) For the CC83 light chain, CC83 CDR2(-):
5'-CAGGGACTCCAGTGTGC 3',

-83-

CC83 L intron (-):

5'GACTTCAAGATACAAATGTTAG-3',

and JK4(-)-20mer:

5'-CCAACTTTGTCCCCGAGCCGAACG.

5 The complete nucleotide sequences for CC49 V_L
and CC83 V_L are shown in Figures 4a and 5a,
respectively.

For the CC49 heavy chain, J_H4 (-)-20mer:

10 5'GGTGACTGAGGTTCCTTGAC-3' a

and J_H4 Intron (-):

5'-GCAATGCTCAGAAAACCTCC.

For the CC83 heavy chain, J_H2(-)-16mer:

15 5'CTGAGGAGACTGTGAG-3'

and J_H2 Intron(-):

5'-GCAGTAAATCTATCTAAGCTG.

20 Thereafter, the sequencing of each heavy chain
was extended with the following sequences: CC49/83
HC/5'(+)

5'-GCACTGCTCATGATATGCAAATC-3';

CC49/83 HC/5'(-)

5'-GATTTGCATATCATGAGCAGTGC-3';

25 and CC49/83 H chain FRI(-)

5'-CTCAGCGTCAGACTGCTG-3'.

30 The complete nucleotide sequences for CC49 V_H
and CC83 V_H are shown in Figure 2.

Comparisons were made between the characterized
mRNA sequence and the characterized DNA sequence, and
between the characterized amino acid sequence with the
amino acid sequence predicted from the DNA sequence.
Based on these comparisons, the plasmid clones were

-84-

identified to contain the correct DNA sequence to code for the CC49 and CC83 heavy and light chain variable regions.

5 The predicted amino acid sequences from the
nucleotide sequences of the heavy chain variable regions
of CC49 and CC83, as shown in Figure 2, show extensive
sequence similarity throughout the framework regions and
hypervariable regions 1 and 2. Hypervariable region 3
10 is quite different between the two due to the
recombination of the V_H region with different D and J_H
sequences, namely that the CC49 $\gamma 1$ heavy chain used a
 J_H^4 , and the CC83 gamma 1 used a J_H^2 .

15 The extensive DNA sequence homology 5' to the
coding regions in the CC49 and CC83 heavy chain variable
region genes shows the two heavy chain variable region
genes were derived from the same germline exons.

20 Isolation of $V_H\alpha$ TAG, Germline Precursor Gene to the
Heavy Chain of CC46, CC49, CC83, and CC92

The procedures used to isolate the germline
precursor gene to the heavy chain variable regions of
CC46, CC49, CC83, and CC92 were essentially those used
25 to isolate the CC49 heavy chain variable region except
that the DNA used to generate the LAMBDA-ZAP™ library
came from an irrelevant hybridoma cell line (i.e., a
cell line which produces antibodies that do not
30 appreciably bind to TAG72). A genomic library
containing approximately 900,000 plaques was screened
from which one positive clone was isolated. The
positive clone was named p $V_H\alpha$ TAG. p $V_H\alpha$ TAG was about 5.2
kb, and the size of the DNA insert was determined by
restriction enzyme mapping to be about 2.2 kb.

DNA sequence of V_HαTAG

The following oligonucleotide primers were used for determining the DNA sequence of V_HαTAG:

B72.3/CC92 HC-20mer: 5'-CCTTGAACCTTCTCATTGTAC-3';

5 CC49/CC83 HC 5'(+): 5'-GCACTGCTCATGATATGCAAATC-3';

CC49/CC83 HC 5'(-): 5'-GATTTGCATATCATGAGCAGTGC-3';

V_HαTAG IVS (+): 5'-CTAAAGTGGAGTCAGGGCCTG-3';

V_HαTAG IVS (-): 5'-CAGGCCCTGACTCCACTTTAG-3';

10 V_HαTAG CDR2 (+): 5'-GAATGGATTGGATATATTTCTC-3'.

The complete nucleotide sequence of V_HαTAG is shown in Figure 2.

Isolation of Human Heavy Constant Genes

15

Plasmid constructs containing the various heavy chain human constant regions (py1, py2, py3, and py4) were provided by Dr. Ilan R. Kirsch of the National Cancer Institute, Bethesda, Maryland.

20

Restriction enzyme mapping was performed on these genes to confirm their identity. Restriction maps for the human constant regions are enclosed in Figure 21.

25

Chimeric Light ChainMurine CC49 V Region

30

The *Hind* III site of the CC49 light chain genomic DNA located in the murine intron region between J5 and C_k (see Max, Edward E. et al., *J. Biol. Chem.* 256, 5116 (1981) was lost in the cloning procedure where half-filled in *Hind* III sites were ligated to half-filled in *Spe* I sites in the LAMBDA-ZAP vector. The plasmid pRL101 (Figure 9) carried this modification.

-86-

5 The intron *Hind* III site was regenerated as outlined in the steps below in order to enable a *Hind* III-*Bam* HI human germline kappa light chain DNA fragment (see Hieter, P. et al., *J.Biol.Chem.* 257, 1516 (1982) to be ligated to the murine variable region directly. All steps were performed using standard molecular biology techniques familiar to artisans and can be found in a manual such as *Manatis*.

10 A 1.69 kb *Bam* HI-*Pst* I fragment is isolated from pRL101, described supra. A 2.96 kb *Bam* HI-*Pst* I fragment is isolated from pBluescript SK(-) (purchased from Stratagene), described supra. The two fragments are then ligated and pRL103, below, is isolated.

15

20

25

30

-87-

Plasmid pGD1, (described supra), was digested with *Pst*I and *Hind*III restriction enzymes to yield the necessary 1.03 kb intron-containing fragment, and pRL103 was also digested with *Pst*I and *Hind*III restriction enzymes to remove the small fragment of DNA in the polylinker.

The resulting fragments were ligated with T4 DNA ligase to produce a 5.68 kb plasmid, called pRL104. A partial restriction map of pGD1 and pRL104 are shown below.

15

20

25

30

-88-

Human C_K Region

Plasmid phum C_K was obtained from Dr. John Roder, Mt Sinai Research Institute, Toronto, Ontario, Canada. The plasmid is derived from pBR322, with a 12 kb *Bam* HI fragment containing the human C_K exon inserted therein. pBR322 is described on page 171 of the 1987 Stratagene catalog. The 12 kb *Bam* HI fragment restriction map is shown below [from Heiter, P. et al., *J.Biol.Chem* 257, 1516 (1982)].

10

15

20

25

30

-89-

The plasmid phum C_k was digested with *Hind* III and *Bam* HI restriction enzymes to yield a 5.0 kb fragment, containing the human C_k exon. pRL104 was digested with *Fsp* I and *Hind* III restriction enzymes to yield a 4.2 kb fragment, containing the mouse light chain variable exons of CC49.

The two resulting fragments were joined with T4 DNA ligase to produce a 9.2 kb fragment among the mixture of resulting fragments. This mixture was digested with *Bam* HI to yield an 7.7 kb *Bam* HI CC49 L chain chimeric construct with *Bam* HI sticky ends, which contains both the mouse variable region exons and the human constant region (kappa) exon. These constructions utilize the human enhancer sequences and the murine promoter sequences.

The chimeric *Bam* HI fragment containing both the murine light chain variable region exons (L and VJ) and the human constant region kappa (k) exon was ligated into the *Bam*HI site of with the plasmid pSV2neo (5.6 kb), a pBR322-derived plasmid containing the selectable marker gene *neo* (obtained from ATCC). The presence of the active *neo* gene renders a cell resistant to growth inhibition by Geneticin, a neomycin-like drug also called G418.

The chimeric *Bam* HI fragment was inserted into pSV2neo in both orientations as shown below. Both transcriptional orientations of the chimeric light chain gene, relative to the *neo* gene, were constructed. Plasmid pSV2neo was linearized at the *Bam* HI site, dephosphorylated (according to procedures set forth in *Maniatis*) using calf intestinal alkaline phosphatase (to

-90-

prevent self-ligation) and ligated with chimeric CC49 L chain *Bam* HI fragments from above.

5

10

15

20

25

30

-91-

The transcriptional orientations of the *neo* gene and the CC49 chimeric light chain are indicated by arrows in pRL150 and pRL105. The portions derived from pSV2neo are indicated. These plasmids were purified on a large scale from preparative scale (1.0L) fermentation of *E. coli* clones replicating each of the plasmids. The purified plasmids were used to introduce the chimeric CC49 light chain into SP2/0 plasmacytoma cells as discussed below.

10 Murine CC83 VL Region and Human C_κ Region

The *Hind* III site in pRL200 which was lost in the cloning process of the CC83 light chain was regenerated for the same reason as for the CC49 light chain chimeric construction. The regeneration was accomplished as follows. The plasmid pRL200 was linearized at a unique *Nhe* I site, and both of its sticky ends were converted to blunt ends by filling in with dNTPs and DNA polymerase I. A *Bam* HI phosphorylated linker (purchased from New England Biolabs) was ligated to the filled-in site. The new plasmid is called pRL201 and is shown below.

25

30

The 2.5 kb *Bam* HI-*Pst* I fragment from pRL201 containing the CC83 light chain variable region genomic DNA was conveniently ligated to the 4kb *Bam* HI-*Pst* I vector fragment from pRL104 which was described earlier in the CC49 light chain constructions and which already
5 had the Hind III-bearing intron fragment. The new plasmid is called pRL202 and is shown below.

10

15

20

25

30

-93-

The approximately 5.05 kb *Fsp*I-*Hind* III fragment from pRL202 was isolated and ligated with the human C_k-containing 5.0 kb *Hind* III-*Bam*HI fragment already described for the CC49 light chain chimeric construction. The generation of the CC83 light chain vector was accomplished from this point in an identical fashion as carried out for the CC49 light chain. The resulting 8.5 kb *Bam* HI CC83 light chain chimeric construct was also ligated to pSV2neo-*Bam* HI (phosphatased) and plasmids with both possible orientations of the insert were obtained as diagramed below.

15

20

25

30

-94-

The transcriptional orientations of the *neo* gene and the CC83 chimeric light chain are indicated by arrows in pRL203 and pRL230. These plasmids were purified on a large scale from preparative scale (1.0L) fermentation in a commercial incubator of *E.coli* clones replicating each of the plasmids. The purified plasmids were used to introduce the chimeric CC83 light chain into Sp2/0 plasmacytoma cells, as discussed later.

All four of the chimeric light chain plasmid constructs (pRL105, pRL150, pRL203 and pRL230) can be linearized by digesting with the restriction enzyme Aat II. The Aat II site in the plasmids is in a region that is not essential for the expression of the chimeric light chain gene or the selectable marker gene, *neo*.

Chimeric Heavy Chains

Human Gamma Constant Gene Exons

The plasmid vector used to carry the chimeric heavy chain constructs is designated pSV2gpt, set forth in Mulligan and Berg, "Selection of animal cells that express the *E.coli* gene coding for xanthine-guanine phosphoribosyltransferase", *Proc.Natl.Acad.Sci(USA)* 78(4), 2072-2076 (1982). pSV2gpt is a pBR322 derived plasmid containing the selectable marker gene, guanine phosphoribosyl transferase (gpt), which can be used for selective growth in media containing mycophenolic acid. To prepare pSVgpt as a recipient for the human C γ 1, C γ 2, C γ 3, C γ 4 exons, it was digested with *Eco* RI and *Bam* HI. The digested DNA was fractionated on a 4 percent polyacrylamide gel and the 4.5 kb vector fragment was recovered from the gel by electroelution as described in *Maniatis*. This linearized plasmid was designated

-95-

pSV2gpt/R/B, a plasmid map is shown in Figure 22. It is able to accept *Eco* RI-*Bam* HI ended fragments.

5 The 5' *Hind* III sites, present on the human IgG₁ constant region fragments, were converted to *Eco* RI sites for directed cloning into the *Eco* RI site of pSV2-gpt. For γ 1, γ 2, γ 3, and γ 4, the *Eco* RI site in vector, pBR322 was employed.

C γ 1

10 The fragment containing the human C γ 1 exons was obtained by digesting and linearizing p γ 1 with *Hind* III followed by filling in the *Hind* III sticky ends using all four dNTP's and the Klenow fragment of DNA
15 Polymerase to make the *Hind* III ends blunt. An *Eco* RI linker was ligated to the blunt ends to replace the *Hind* III site with an *Eco* RI site. This construct was then digested with *Eco* RI and *Bam* HI to release a 7.8 kb fragment containing the C γ 1 exons. This fragment was
20 called C γ 1-7.8 kb.

The fragment were each ligated into the *Eco* RI-*Bam* HI sites of pSV2-gpt/R/B. This vector (pSV2-gpt- γ 1-7.2) design allows us to insert any murine heavy chain
25 variable region gene (with *Eco* RI ends) into the *Eco* RI site of the human IgG heavy chain vectors. More specifically, 125 ng of the human C γ 1-7.8 kb fragment was ligated to 100 ng of the linearized pSV2gpt/R/B
30 vector in a volume of 10 μ l using 400 units of T4 DNA ligase (obtained from New England Biolabs). Frozen competent *E.coli* DH1 cells from Invitrogen (San Diego, CA) were transformed with a ligation reaction according to the Invitrogen's protocol. The resulting plasmid was

-96-

designated pSV2gpty1-7.8. A plasmid map of pSV2gpty1-7.8 is shown in Figure 23.

In addition, another shorter fragment containing the Cy1 exons was generated. Concerns about the total size of the chimeric heavy chain vector, with a 7.8kb Cy1 fragment, a 4.5kb pSV2-gpt/R/B vector, and a CC49 variable region of 1.9kb (total=14.2kb) prompted the need to reduce the large size of the 7.8kb Cy1 *Eco* RI-*Bam* HI fragment. The coding region of 7.8kb Cy1 occupies only the first 1/3 of the 5' end of the fragment.

Size reduction was accomplished by converting a downstream *Pvu* II site to a *Bam* HI site by blunt-end addition of a *Bam* HI linker. The *Hind* III site of p γ -1 was converted to an *Eco* RI site by digestion of p γ -1 with *Hind* III, filling in the 3' end to create a blunt end, and addition of *Eco* RI linkers as above. The *Pvu* II site 2.3 kb downstream was converted to a *Bam* HI site by subsequent digestion with *Pvu* II and ligation of *Bam* HI linkers directly to the blunt *Pvu* II ends. This construct was then digested with *Eco* RI and *Bam* HI to release a 2.3 kb fragment containing the Cy1 exons. The shortened *Eco*RI-*Bam*HI fragment (2.3 kb) still contains the γ 1 exons and the 3' polyadenylation sequence. This reduces the total vector size by 5.5kb, making the overall construct more manageable (total=8.7 kb).

Approximately 200 ng of the human Cy1 2.3 kb fragment was ligated to 100 ng of the linearized plasmid pSV2gpt/R/B vector in a volume of 10 μ l using 400 units of T4 DNA ligase (New England Biolabs). Frozen competent *E.coli* cells, obtained from Invitrogen, were transformed with the ligation reaction according to

-97-

Invitrogen's protocol. The resulting plasmid was designated pSV2gpt γ 1-2.3. A plasmid map of pSV2gpt γ 1-2.3 is shown in Figure 24.

DNA fragments containing the other three human IgG constant region exons were also isolated. The C γ 2 exons were recovered from the plasmid p γ 2 as a 4.0 kb *Eco* RI-*Bam* HI fragment. The C γ 3 exons were recovered from the plasmid p γ 3 as an 8.0 kb *Eco* RI-*Bam* HI fragment. The C γ 4 exons were recovered from the plasmid p γ 4 as a 7.6 kb *Eco* RI-*Bam* HI fragment. The fragments were separately ligated into pSV2gpt/R/B as described for C γ 1-7.8 and C γ 1-2.3. Plasmid maps of the resultant plasmids are shown in Figure 25, pSV2gpt- γ 2; Figure 26, pSV2gpt- γ 3; and Figure 27, pSV2gpt- γ 4.

Heavy Chain Chimeric Constructs:

The complete heavy chain variable region human γ 1 constant region chimeric constructs were generated by inserting a fragment containing the murine heavy chain variable region exons into the plasmids containing the human γ 1 constant region exons described as follows.

Eco RI fragments containing the murine heavy chain variable region genes from CC49 and CC83 hybridoma cells were then ligated into each of the γ 1- γ 4-containing pSV2-gpt vectors (pSV2gpt- γ 1; pSV2gpt- γ 2; pSV2gpt- γ 3; pSV2gpt- γ 4) as follows.

CC49

A fragment containing the heavy chain variable region exons coding for the CC49 heavy chain variable region was prepared by digesting 14 μ g of pHH49 with 50 units of *Eco* RI (obtained from BRL) at 37°C for 2 hours.

-98-

The digest was fractionated on a 4 percent polyacrylamide gel and the 1.9 kb *Eco* RI fragment containing the heavy chain variable region exons of CC49 was recovered by electroelution as described by *Maniatis*. This fragment was designated f49R.

5

A fragment containing the 7.8 kb sequence encoding for γ 1 was prepared as follows.

Approximately 50 μ g of the vector pSV2gpt γ 1-7.8 was digested with *Eco* RI. The resultant fragment was dephosphorylated (to prevent self ligation) using calf intestinal alkaline phosphatase as described by *Maniatis*. The fragment was purified from the 0.8 percent agarose gel by electroelution. This vector was designated pSV2gpt γ 1-7.8/R.

10
15

The *Eco*RI site is located 245 bp upstream of the transcription initiation sites, and contains the promoter and the necessary tissue-specific sequences for efficient expression. The intron regions 3' of the variable region genes contain the murine heavy chain enhancer sequences which are absent on the human IgG heavy chain vectors. Therefore, the heavy chain chimeric vectors use both murine promoter and enhancer sequences.

20
25

Approximately 325 ng of linearized pSV2gpt γ 1-7.8/R was ligated with 188 ng of f49R in a volume of 10 μ L with 1 unit of T4 DNA ligase (BRL). Frozen competent *E. coli* AG-1 cells from Stratagene were transformed with the ligation reaction according to their protocol. The resulting plasmid was designated p49 γ 1-7.8. Figure 28 illustrates a plasmid map for p49 γ 1-7.8.

30

Approximately 50 μ g of the vector pSV2gpt γ 1-2.3 was digested as for SV2gpt γ 1-7.8 with *Eco* RI. The

-99-

resultant fragment was dephosphorylated using calf intestinal alkaline phosphatase as described by *Maniatis*. The fragment was purified from an 0.8 percent agarose gel by electroelution. This linearized plasmid was designated pSV2gpt γ 1-2.3/R.

5

Approximately 300 ng of the linearized plasmid pSV2gpt γ 1-2.3/R was ligated with 188 ng of f49R in a volume of 10 μ l with 1 unit of T4 DNA ligase (BRL). Frozen competent *E. coli* AG-1 cells from Stratagene (La Jolla, CA) were transformed with the ligation reaction according to their protocol. The resulting plasmid was designated p49 γ 1-2.3. Figure 29 illustrates a plasmid map for p49 γ 1-2.3.

10

15

Plasmids pSV2gpt- γ 2, pSV2gpt- γ 3 and pSV2gpt- γ 4 were separately digested with *Eco* RI to produce the linear plasmid vectors pSV2gpt- γ 2/R, pSV2gpt- γ 3/R and pSV2gpt- γ 4/R respectively. Each of these 3 linear plasmid vectors were separately ligated with f49R. Plasmid maps of the resulting plasmids are shown in Figure 30, p49- γ 2; Figure 31, p49- γ 3; and Figure 32, p49- γ 4.

20

25

CC83

Chimeric constructs containing the heavy chain variable region of CC83 were generated in a similar manner as the chimeric constructs of CC49. A fragment containing the heavy chain variable region exons coding for the CC83 heavy chain region was prepared by digesting 19 μ g of pHS83 with 50 units of *Eco* RI (obtained from BRL) at 37°C for 2 hours. The digest was fractionated on a 4 percent polyacrylamide gel and the 2.9 kb *Eco* RI fragment containing the heavy chain

30

-100-

variable region exons of CC83 were recovered by electroelution as described in *Maniatis*. This fragment was designated f83R.

Approximately 300 ng of the linearized plasmid pSV2gpt γ 1-7.8/R, obtained as above, was ligated with 270 ng of f83R in a volume of 10 μ l with 1 unit of T4 DNA ligase (obtained from BRL). Frozen competent *E. coli* AG-1 cells, obtained from Stratagene, were transformed with the ligation reaction according to Stratagene's protocol. The resulting plasmid was designated p83 γ 1-7.8. Figure 33 illustrates the plasmid map of p83 γ 1-7.8.

Approximately 90 ng of linearized plasmid pSV2gpt γ 1-2.3/R, obtained as above, was ligated with 270 ng of f83R in a volume of 10 μ l with 1 unit of T4 DNA ligase (BRL). Frozen competent *E. coli* AG-1 cells from Stratagene were transformed with the ligation reaction according to their protocol. The resulting plasmid was designated p83 γ 1-2.3. Figure 34 illustrates the plasmid map of p83 γ 1-2.3.

Plasmids pSV2gpt- γ 2, pSV2gpt- γ 3 and pSV2gpt- γ 4 were separately digested as above for pSV2gpt- γ 2/R, pSV2gpt- γ 3/R and pSV2gpt- γ 4/R, respectively, with *Eco* RI to produce the linear plasmid vectors pSV2gpt- γ 2/R, pSV2gpt- γ 3/R and pSV2gpt- γ 4/R respectively. Each of these 3 linear plasmid vectors were separately ligated with f83R. Plasmid maps for the resulting plasmids are shown in Figure 35, p83- γ 2; Figure 36, p83- γ 3; and Figure 37, p83- γ 4.

All ten of the circular plasmid constructs (p49 γ 1-7.8; p49 γ 1-2.3; p83 γ 1-7.8; p83 γ 1-2.3, p49- γ 2;

p83- γ 2; p49- γ 3; p83- γ 3; p49- γ 4; and p83- γ 4) were then linearized for transformation by digesting with the restriction enzyme *Nde* I. The *Nde* I site in the plasmids is in a region that is not essential for the expression of the chimeric immunoglobulin gene or the selectable marker gene, gpt. The plasmids need to be in a linear form prior to transformation into a recipient cell to enhance selected integration of the DNA into the host cell genomic DNA.

10 Verification of Construction

Since the *Eco*RI fragments can be ligated in either orientation, the correct orientation was determined by digestion with *Nco* I. In the constructions set forth above, correct ligations for plasmid construction are confirmed by performing restriction enzyme site mapping on the plasmid. The restriction enzyme map generated from restriction enzyme digestion and gel electrophoresis is compared to that which can be theoretically generated from the individual starting fragments. Because of the experience with the transcriptional orientation in the light chain vectors, the heavy chain vectors were constructed only in the opposite transcriptional orientation to the gpt gene.

Transformation of Plasmids into Mouse Plasmacytoma Cells

When both light chain and heavy chain chimeric genes were transformed into the same cell, tetrameric (H₂L₂) immunoglobulins are obtained. Synthesis and secretion of these "chimeric" antibody proteins was accomplished by introducing the chimeric (mouse V:human C region) genes into mouse plasmacytoma cells(Sp2/).

Transformation was achieved by electroporation [Sahagan, B.G. et al., *J.Immunology* 137, 1066 (1986)].

Expression of chimeric (mouse V:human C region) genes in transformed mouse plasmacytoma cells (Sp2/0) is achieved using two different techniques. In one mode, different ratios of light chain genes to heavy chain genes can be introduced together. This is referred to as cotransformation. Alternatively, stable clones carrying the chimeric light chain gene can be obtained and subsequently used in a second mode referred to as targeted transformation. In each method, the goal is to obtain clones containing genes for both the H chain and L chain which produce intact H₂L₂ immunoglobulin mentioned above.

A. Cotransformations

Co-transformation involves the transformation of cells with both drug resistance markers at the same time and subsequent selection with one or both drugs. Co-transformation of heavy chain and light chain vectors (at ratios of 1:1 and 1:10, respectively) was originally performed using only neo selection. Neo-resistant cell lines were obtained which expressed the first chimeric IgG1 antibodies with demonstrable TAG72 binding activity. Cotransformation was conducted pursuant to the protocols set forth in Cornelia Gorman, "High Efficiency Gene Transfer into Mammalian Cells", DNA Cloning, Vol II, D. M. Glover ed, IRL Press, Oxford, England (1985).

B. Targeted Transformations

Constructs containing light and heavy chimeric immunoglobulin genes were sequentially transformed using into Sp2/0 mouse plasmacytoma cells. Targeted transformation involves transformation and selection with a vector containing a first drug-resistance gene (i.e., Geneticin for the chimeric light chain gene vector), followed by transformation and selection with a vector containing a second drug resistance gene (i.e., mycophenolic acid for the chimeric heavy chain gene vector).

Neo Selection

Prior to transformation with pSV2-neo vectors, which contain chimeric light chain constructions, drug selection conditions for inhibition of growth of untransformed Sp2/0 plasmacytoma cells [obtained from the American Type Culture Collection (ATCC)] were established by titration of the neomycin-like drug, Geneticin (GIBCO). Published values for concentrations of Geneticin used for drug selection ranged from 100-1000 µg/mL. Concentrations above 400 µg/mL were found to prevent growth of Sp2/0 cells in our tissue culture environment.

Construction of Light Chain Containing Cells

Sp2/0 mouse plasmacytoma cells were initially transformed with light chain-containing pSV2-neo vectors as follows. Cells were grown in RPMI 1640 medium with 5 percent fetal calf serum. Cells were washed in PBS and suspended to a concentration of 1×10^7 viable cells/mL PBS. 0.8 mL of cells were transferred to an

-104-

electroporation curvette (on ice) containing 20 µg of light chain-containing pSV2-neo vector (pRL105 and pRL150 for the CC49 chimeric L chain and pRL203 and pRL230 for the CC83 chimeric L chain) linearized with Aat II restriction endonuclease. Aat II was inactivated by heating the samples to 65°C for 10 minutes. The linearized DNA was ethanol precipitated and subsequently dissolved in 10-20 microliters of PBS. After 15 minutes on ice, electroporation was performed using a Gene Pulser electroporation apparatus with added capacitance extender (BioRad) at 0.2 kvolts and 960 µF. The time constant (τ) was generally about 26 msec.

After transformation, cells were allowed to recover on ice for 15 minutes to allow relaxation of perturbed membranes. Afterwards, the cells were suspended in 24 mL of RPMI 1640 medium containing 5% fetal calf serum (RPMI+) and transferred to a 96 or 24 well plate. To decrease the probability of more than one drug resistant cell per well, the cells were also diluted 10-fold in medium (RPMI+) and plated into another 96-well (or 24 well) plate. The cell suspension was incubated at 37°C and 5 percent CO₂ atmosphere.

After 48 hours (to allow for expression of drug resistance), the medium was removed and replaced with medium containing 1 mg/mL Geneticin.

After 7-10 days, Geneticin-resistant clones were subcultured and the cells screened for chimeric light chains by cytostaining.

Cytostaining

Aliquots of cells were pelleted onto a glass slide using a cytospin-2 centrifuge (Shandon, Inc.).

After air drying, the cells were fixed in acetic acid/ethanol (5 parts acetic acid/95 parts ethanol). After rinsing 3 times with PBS (without CA^{+2} and Mg^{+2}), the slides were placed in a humid chamber (100% RH), and stained for 20 minutes with 20 μ l of goat anti-human
5 Kappa-FITC, a fluorescent dye-conjugated antibody which is specific for human kappa light chains. The conjugated antibody was diluted 1:3 with 1% BSA in PBS. After washing overnight with PBS, the slides were
10 mounted with fluoromount-G, histologic mounting medium (obtained from Southern Biotech) under a coverslip. The slides were observed with an Olympus model BH-2 microscope equipped with an epi-illumination U.V. attachment.

15 Based on the intensity of fluorescence, the constructions with the orientation of the light chain in opposite transcriptional orientation relative to the direction of transcription of the neo^r gene in the
20 vector, was found to give the highest L chain expression. Therefore, pRL105 was the preferred CC49 L chain construction and pRL230 was the favored CC83 L chain construction. As a result of these experiments
25 the following chimeric light chain-containing cell lines (derived from Sp2/O) were used for the targeted transformations:

For the CC49 chimeric L chain one cell line
30 (49K-13-13) was obtained which expressed the chimeric light chain derived from CC49. This cell line was used for all subsequent targeted transformations with chimeric heavy chain vectors for constructs using the chimeric CC49 light chain.

-106-

For the CC83 chimeric L chain three cell lines (83K-26-5, 83K-34-10, and 83K-42-2) were obtained which expressed the chimeric light chain derived from CC83. One cell line (83K-26-5) stained more intensely than the others and had localized regions of cytoplasmic immunofluorescence. All three cell lines were compared for their relative ability to produce high levels of chimeric antibody after transformation with the chimeric CC83 g1 heavy chain vector. More clones expressing chimeric antibodies were derived from electroporation of the 83K-34-10 target than either of the other two chimeric light chain target cell lines. Therefore, the 83K-34-10 light chain cell line was used as a target for subsequent electroporations with chimeric heavy chain vectors for constructs containing the CC83 light chain variable region.

Generation of *gpt* Resistant clones carrying CC49 and CC83 chimeric H chain constructions

Prior to transformation with pSV2-*gpt* vectors, which contain chimeric heavy chain constructions, drug selection for inhibition of growth of untransformed Sp2/0 plasmacytoma cells [obtained from the American Type Culture Collection (ATCC)] were established. Conditions for drug selection of cells transformed with pSV2-*gpt* vectors were more difficult to establish. The *E. coli gpt* gene, which codes for the enzyme guanosine phosphoribosyl transferase, confers the ability to utilize xanthine and hypoxanthine as substrates for the biosynthesis of guanine when the mammalian guanine metabolic pathway is inhibited by mycophenolic acid (MPA).

-107-

Published values for the concentrations of MPA which allow for the growth of other lymphoid cell lines transformed with pSV2-gpt vectors were found to be almost two orders of magnitude too high to allow for the growth of Sp2/0 cells transformed with pSV2-gpt vectors in our tissue culture environment. Subsequently, a concentration of 0.1ug/mL of MPA was found to be optimal for selection of gpt resistance. In addition, the use of aminopterin and thymidine (to further shut down the guanine pathway) was found to be unnecessary.

Generation of Clones Producing of Chimeric 44 Antibody

CH44-1

49K-13-13 cells were used as a target for chimeric heavy chain constructs. The cells were transformed with 20 µg chimeric heavy chain DNA vector (p49γ1-7.8 or p49γ1-2.3) linearized by *Nde* I digestion. Transformation by electroporation was performed as above for chimeric light chains.

Selection after 48 hours, however, was performed by replacing the geneticin-containing medium with medium containing geneticin and 0.3 µg/mL mycophenolic acid, 250 µg/mL xanthine, and 10 µg/mL hypoxanthine.

Transformed cells grew to macroscopically visible colonies in 14 days. At that time, 50ul of supernatant was removed and assayed by ELISA methods for binding to TAG and expression of human IgG constant region. Wells containing cells with positive TAG binding were expanded to 24-well plates with fresh drug selection medium and allowed to grow for 3-7 days.

Subcloning was performed as follows. Viable cell counts were determined and the cells were replated into two 96-well plates. One plate received 50 viable cells and the other received 250 viable cells. The unsubcloned cells were expanded to 6-well plates until
5 the cell density was sufficient to allow for storage in liquid nitrogen in the event that re-subcloning would be necessary.

After subcloning, those clones exhibiting the
10 highest chimeric antibody production were selected for chimeric antibody production in bioreactors.

CH44-2

15 The procedures used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of CH44-1 were repeated with the exception that 20 µg of p49-γ2, was used as the chimeric heavy chain vector.

20 CH44-3

The procedures used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of CH44-1 were repeated with the exception that 20 µg of
25 p49-γ3, was used as the heavy chain vector.

CH44-4

The procedures used to sequentially transform
30 the Sp2/0 plasmacytoma cells in the construction of CH44-1 were repeated with the exception that 20 µg of p49-γ4, was used as the heavy chain vector.

Generation of Clones Producing of Chimeric 88 Antibody

CH88-1

5 The procedures used to sequentially transform
the Sp2/0 plasmacytoma cells in the construction of
CH44-1 were repeated with the following exceptions:

83K-26-5, 83K-34-10, and 83K-42-2 cells
demonstrating production of chimeric CC83 light chain
10 were transformed as described in the transformation of
CH44-1, with the exception that 20 µg of p83γ1-7.8 or
p83γ1-2.3, the pSV2gpt vector which contains the
chimeric CC83 heavy chain gene was used as the heavy
chain vector.

15 CH88-2

 The procedures used to sequentially transform
the Sp2/0 plasmacytoma cells in the construction of
20 CH88-1 were repeated with the exception that 20 µg of
p83-γ2, was used as the heavy chain vector.

25 CH88-3

 The procedures used to sequentially transform
the Sp2/0 plasmacytoma cells in the construction of
CH88-1 were repeated with the exception that 20 µg of
30 p83-γ3, was used as the heavy chain vector.

CH88-4

 The procedures used to sequentially transform
the Sp2/0 plasmacytoma cells in the construction of

CH88-1 were repeated with the exception that 20 µg of p83-γ⁴, was used as the heavy chain vector.

Generation of Clones Producing of Chimeric 84 Antibody

5 Because of the high degree of sequence
similarity between the heavy chain variable regions of
CC49 and CC83, chimeric antibodies were generated whose
light and heavy chains were derived from different
parents by mixed targeted transformations. To generate
10 both "mixed" combinations, the chimeric heavy chain γ¹
isotype vectors of CC49 and CC83 were electroporated
into the chimeric light chain targets 83K34-10 and 49K-
13-13 respectively. The resulting cell lines were
designated CH48-1 and CH84-1, where the first numerical
15 designation represents the heavy chain and light chain
parents, respectively. For example, CH48-1 represents
the γ¹ isotype with the heavy chain derived from CC49
and the light chain derived from CC83.

20 The CH48-1 composite antibody did not bind to
TAG72. This was not due to the inability to make bona
fide chimeric antibody, since most drug-resistant cell
lines produced chimeric IgG (as determined by ELISA
25 analysis using Goat Anti-Human Ig trap with Goat Anti-
Human IgG-Alkaline Phosphatase as a probe). If any
binding affinity were present, it was significantly less
than that observed for the first generation antibody
B72.3, which was approximately an order of magnitude
30 less affinity for TAG72 than either CC49 or CC83.

Surprisingly, CH84-1 bound to TAG72 with affinity similar to both parents. This new antibody was generated de novo in our laboratory and has not yet been detected in nature.

5 Competition studies were undertaken to determine the specificity of this new mixed-antibody, CH84-1. It should be noted that both CC49 and CC83 exhibit some competitive recognition for the TAG72 antigen. It was found that CH84-1 competed more with
10 CC49 for binding to TAG72 than it did with CC83. This would indicate that the specificity for binding to TAG72 lies in the light chain.

 Human γ 2, -3, and -4 isotypes were also
15 generated with this mixed-antibody, producing CH84-2, CH84-3, CH84-4 clones.

CH84-1

20 The procedure used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of CH44-1 were repeated with the following exception:

 49K-13-13 cells demonstrating production of
25 CH44 light chain by cyto staining were then transformed as described in the transformed of CH44-1, with the exception that 20 μ g of p83 γ 1-2.3, the pSV2gpt vector which contains the CH83 heavy chain gene was substituted
30 for p49 γ 1-2.3, the pSV2gpt vector which contains the CH44 heavy chain gene.

CH84-2

 The procedures used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of

-112-

CH84-1 were repeated with the exception that 20 µg of p83-γ2, was substituted for p83γ1-2.3.

CH84-3

5 The procedures used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of CH84-1 were repeated with the exception that 20 µg of p83-γ3, was substituted for p83γ1-2.3.

10 CH84-4

 The procedures used to sequentially transform the Sp2/0 plasmacytoma cells in the construction of CH84-1 were repeated with the exception that 20 µg of p83-γ4, was substituted for p83γ1-2.3.

Purification of Recombinant Antibodies

20 Cells expressing the chimeric antibodies were removed by centrifugation from the culture medium and the medium was filtered through a 0.2µm filter. Chimeric antibodies were purified in two steps from culture supernatants. In the first step of the purification, a protein A affinity cartridge (Nygene Corporation, Yonkers, NY) was utilized according to the manufacturer's specifications. Up to 1.0 L of culture supernatant was passed through a 1 mg capacity cartridge, at 5 mL/min. The cartridge was washed with phosphate buffered saline (PBS) to remove traces of albumin. The chimeric antibody was recovered by elution with 0.1M sodium nitrate buffer, pH 3.0. The pH of the fractions containing the chimeric antibody were immediately adjusted to neutrality with a 1M solution of Trizma base. Final purification was achieved from this solution, after concentration on an Amicon centricon 30

-113-

unit, by gel filtration using a Pharmacia Superose 12 HR 16/50 column as specified by the manufacturer (Pharmacia, Piscataway, NJ).

EXAMPLE: Generation of an Immunoglobulin Containing the
5 Murine V_HαTAG germline Variable Region

The following examples are set forth to provide a skilled artisan with a reproducible technique for preparing an antibody having a V_H region encoded by a
10 DNA sequence derived from V_HαTAG.

Components for an Expressible V_HαTAG Heavy Chain Gene

A mouse-human chimeric antibody molecule can be
15 generated which contains the murine V_HαTAG germline heavy chain variable region, a light chain variable region that is complementary to the V_HαTAG V_H, such as either the CC49 or CC83 murine light chain variable region, and human constant regions.

20 The 2.2 kb HindIII germline DNA fragment containing the V_HαTAG V_H exon sequence is used as a template to obtain a functionally rearranged V_HαTAG variable region. The murine genomic J-C_μ intron region
25 is used as a source for the murine heavy chain enhancer sequences. This latter region is obtained from the plasmid pNP9 (see example above on "Isolation of CC49 Heavy Chain Variable Region"). Figure 38 shows the overall reaction for the engineering of hybrid genes
30 based on the method of Horton et al., (1989), supra. Four oligonucleotides (oligos) are designed to be used in enzymatic amplification and modification of the target DNA. Oligo 1 anneals to the 5' end of V_HαTAG spanning the *Eco*RI site which is 249 bp 5' to the ATG initiation codon. Oligo 2 anneals to sequences

-114-

complementary to the 3' end of the V_HαTAG exon and also contains sequences coding for a D segment. The D segment sequences in oligo 2 do not anneal with any V_HαTAG sequences. Oligo 3 contains sequences complementary to the 5' end of the murine genomic J-Cμ region and incorporates sequences encoding the D segment (same as in oligo 2) and the J segment. Oligo 4 anneals to the 3' end of the J-Cμ region and contains sequences complementary to the *EcoRI* site located 1219 bp 3' to J_H⁴. The sequence of these oligos follow:

Oligo 1 5'GTCTAGAATTCATAAAAACTTTATG (25 mer)

15 Oligo 2 CAGTGTATTTCTGTAAAAGATCTACTATGGTTACG (35 mer)

Oligo 3 5'TCTACTATGGTTACGTGGGGTCAAGGAACCTCAGTCACC
20 GTCTCCTCAGGTAAGAATGGCCTCTCCAGGTCT 3' (72 mer)

Oligo 4 5' ACTTCTAAAATGTATTTAGAATTTCATTTTC 3'

25

In this example, the D sequence is SP2.3 taken from the published sequence of Kurosawa and Tonegawa *J. Exp. Med.*, 155:201 (1982). The D sequence is shown in bold face type in oligos 2 and 3. Any other characterized murine or human D segment can be used by substituting their sequence in these positions of oligo 2 and 3.

30

The J segment in oligo 3 is underlined. It is the murine J_H⁴ taken from the published sequence of Gough and Bernard *Proc.Natl.Acad.Sci.(USA)*, 78:509 (1981). The inclusion of any other murine or human J segment can be made by substituting their sequences for the sequence of J_H⁴ in oligo 3.

In oligo 1 and 4 the *EcoRI* sites (*GAATTC*) are shown in italics.

10 Assembly of Intact V_HαTAG Genes

Two separate DNA amplification reactions are performed using the components described above. DNA amplification reaction #1 copies the V_HαTAG sequence and adds a D segment to its 3' end. DNA amplification reaction #2 copies the murine intron sequences containing the heavy chain enhancer sequences and adds the D and J segments encoded within oligo 3. The amplified products from reaction 1 and 2 are gel purified, combined and oligos 1 and 4 are added to initiate reaction #3. In reaction 3, the products of reactions 1 and 2 anneal across their common D sequences. Subsequent DNA amplification from oligos 1 and 4 yields the product shown at the bottom of Figure 38. This fragment is digested with *EcoRI* and gel purified. The modified V_HαTAG fragment is ligated into the *EcoRI* site of pSV2gptγ1(2.3) as described in the above example "Heavy Chain Chimeric Constructs". The entire V_HαTAG-D-J-enhancer containing fragment is sequenced completely to ensure that no mutations have been introduced during the DNA amplification reactions. The other three heavy chain γ isotypes can be generated by ligating the same modified V_HαTAG fragment into the

other three γ containing pSV2gpt vectors (pSV2gpt- γ 2; pSV2gpt- γ 3; pSV2gpt- γ 4).

5 Expression of the Modified $V_H\alpha$ TAG Gene

 The modified $V_H\alpha$ TAG gene containing plasmids can be linearized with *Nde*I and introduced via electroporation into the chimeric CC49 or CC83 light chain expressing cell lines (see example above, "C. Targeted Transformations". The transformed cells are selected for growth in the presence of Geneticin and mycophenolic acid as outlined above in "C. Targeted Transformations". The presence of expressed antibody is monitored by TAG72 ELISA (see section in RESULTS, Enzyme-Linked Immunoassays (ELISA). The expressed antibody from these cells will contain human Ig γ 1, κ constant regions with the CC49 or CC83 light chain variable region and a heavy chain variable region from the modified $V_H\alpha$ TAG germline V_H exons.

 Four examples of modified $V_H\alpha$ TAG heavy chain variable region constructs having a variety of D and J segments are shown below;

25

<u>V_H Segment</u>	<u>D Segment</u>	<u>J Segment</u>
$V_H\alpha$ TAG #i	mouse D (SP2.3)	mouse J
30 $V_H\alpha$ TAG #ii	human D (D1)	mouse J
$V_H\alpha$ TAG #iii	mouse D (SP2.3)	human J
$V_H\alpha$ TAG #iv	human D (D1)	human J

The sequence of the human D sequence D1 is obtained from Siebenlist et al., *Nature*, 294:631 (1981). The sequence of the human J_H1 is obtained from Ravetch et al., *Cell* 27, 583 (1981).

5 The generation of V_HαTAG #i is described with
the above diagramed oligos 1 through 4. To generate
V_HαTAG #ii through -iv the corresponding D and J
segments need to be changed in oligos 2 and 3. The
10 following oligos delineate these changes. Substitution
of these oligos in reaction #1 and reaction #2 will
result in the generation of the V_HαTAG #ii through -iv.

V_HαTAG #ii

15 Oligo 2 5' CAGTGTATTTCTGTAAAAGAGTACTGGTGGT (34 mer)
 GTAT

Oligo 3 5' GTACTGGTGGTGTATTGGGGTCAAGGAACC (72 mer)
 TCAGTCACCGTCTCCTCAGGTAAGAATGGCCT
20 CTCCAGGTCT 3'

V_HαTAG #iii

Oligo 2 5' CAGTGTATTTCTGTAAAAGATCTACTATGG (35 mer)
25 TTACG

Oligo 3 5' TCTACTATGGTTACGTGGGGCCAGGGCAC (72 mer)
 CCTGGTCACCGTCTCCTCAGGTAAGAATGGCCTCTCCAGGTCT
30 3'

V_HαTAG #iv

Oligo 2 5' CAGTGTATTTCTGTAAAAGAGTACTGGTG (35 mer)
GTGTAT

5 Oligo 3 5' GTACTGGTGGTGTATTGGGGCCAGGGCAC (72 mer)
CCTGGTCACCGTCTCCTCAGGTAAGAATGGC
CTCTCCAGGTCT 3'

10

15

20

25

30

Results

A. Chimeric Antibody - Producing Cell Lines

- 5 Simultaneous detection of heavy and light chains was accomplished using two probe antibodies:
- 1) Goat anti-human kappa labeled with the fluorescing dye FITC and;
 - 10 2) Goat anti-human IgG labeled with the fluorescing dye TRITC.

Cell lines having positive responses for both heavy and light chains were tested further for associated chimeric immunoglobulin production and biological activity viz. binding to TAG72.

Enzyme-Linked Immunoassays (ELISA)

- 20 In order to select a transformed cell producing a chimeric monoclonal antibody, the ELISA technique was employed. Clones containing the heavy chain and light chain drug selection constructs were selected by their growth in selective culture medium. The following cell
- 25 lines were tested (1) CH44-1: a cell line having CC49 V_H, CC49 V_L, and constant region of IgG₁; (2) CH44-2: a cell line having CC49 V_H, CC49 V_L, and constant region of IgG₂; (3) CH44-4: a cell line having CC49 V_H, CC49
- 30 V_L, and constant region of IgG₄; (4) CH88-1: a cell line having V_H, CC83 V_L, and constant region of IgG₁; (5) CH88-2: a cell line having CC83 V_H, CC83 V_L, and constant region of IgG₂; (6) CH88-3: a cell line having CC83 V_H, CC83 V_L, and constant region of IgG₃; (7) CH88-4: a cell line having CC83 V_H, CC83 V_L, and constant

region of IgG₄; (8) CH84-1: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₁; (9) CH84-2: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₂; (10) CH84-3: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₃; and (11) CH84-4: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₄.

Supernatants of these cultures were subjected to ELISA. The presence of chimeric anti-TAG72 antibody was measured directly by reaction of an excess of goat anti-human IgG antibody labeled with an enzyme such as alkaline phosphatase, after allowing the chimeric anti-TAG72 antibody to bind to microtiter wells coated with antigen (TAG72). Anti-TAG72 activity was determined as a criterion for successful recombination.

After growth for 14 days, 50 µl of supernatant was removed from the wells of the subcloned cells and re-assayed for TAG binding by ELISA. Samples of supernatants (50 µl) from drug resistant cell lines were applied to wells of Nunc Immulon 96-well plates which had previously been coated with TAG antigen (1/50 dilution). After washing to remove unbound material, the wells were incubated with Goat Anti-Human IgG antibodies conjugated with Alkaline Phosphatase (GAHIgG-AP) as a probe to detect the human constant regions of the chimeric antibodies which had bound to the TAG antigen immobilized on the plate. Another washing to remove unbound probe (GAHIgG-AP), followed by addition of a chromogenic alkaline phosphatase substrate, allowed color to develop in those wells which possessed TAG binding associated with human constant regions (i.e., chimeric anti-TAG72 antibodies). Absorbance readings at

405 nm indicate the relative amount of chimeric antibody produced by the drug-resistant cell lines.

CH44-1

5 Anti-TAG72 activity was used as a criterion for
successful recombination. Wells of microtiter plates
were coated with TAG by incubating 50 μ l of a 1:75
dilution of purified TAG72 [Muraro, R., et al., *Cancer*
10 *Research* 48, 4588-4596 (1988)] for 18 hours at room
temperature. The wells were then washed 4 times with
phosphate buffered saline (PBS), and then blocked with
BSA, by incubating 50 μ l of 0.5 percent BSA in PBS for 2
hours at 37°C, followed by washing 4 times with PBS.
15 These plates are stable if kept moist at 4°C. 50
microliters of sample are then applied to each well. A
blank containing fresh medium is used as a control. All
of the samples were incubated either in the plate for 90
minutes at 37°C or overnight at 4°C in a closed
20 container.

The plates were then washed 4 times with PBS,
and goat anti-human IgG-alkaline phosphate (Southern
Biotech Assoc.) was applied to each well by adding 50 μ l
25 of a 1:250 dilution. The solution was incubated at 37°C
for 90 minutes. Color development was monitored after
washing the plates 4 times with PBS to remove the probe.

The substrate was incubated in 200 μ l solution
30 of substrate p-nitrophenyl phosphate (Kirkegaard &
Perry) in ethanolamine buffered saline for 6 minutes at
room temperature for color development. The optical
density at 450 nm of each well was read by a Dynatech
microplate reader (Dynatech Inc.).

-122-

The Sp2/0 colonies in wells with supernatants having TAG72-binding chimeric antibody activity were subcloned by limited dilution. Individual subclones were chosen on the basis of relatively high production of chimeric antibody.

5

CH44-2

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH44-

10 2.

CH44-3

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH44-

15 3.

CH44-4

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH44-

20 4.

CH88-1

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH88-

25 1.

CH88-2

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH88-

30 2.

-123-

CH88-3

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH88-3.

5

CH88-4

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH88-4.

10

CH84-1

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH84-1.

15

CH84-2

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH84-2.

20

CH84-3

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH84-3.

25

CH84-4

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH84-4.

30

CH48-1

The TAG-ELISA procedure used with CH44-1 was repeated with the exception that the antibody was CH84-4.

5

B. In Vivo Carcinoma Targeting

The chimeric monoclonal antibodies used in animal studies and shown in Tables 1- 4 below were labeled with Na¹²⁵I using Iodogen (Pierce Chemical, Rockford, IL). More specifically, from about 0.5-2 mg of purified chimeric monoclonal antibodies were adjusted to about 0.5 mL 0.1M sodium phosphate buffer (pH 7.2) and then added to a 12 cm x 75 cm glass tube coated with 50 µg of Iodogen followed by addition of from 0.1 -0.5 mCi of Na¹²⁵I (New England Nuclear, Boston, MA). After a 2 min incubation at room temperature, the protein was removed from the insoluble Iodogen, and the unincorporated ¹²⁵I was separated from the antibody by gel filtration through a 10 mL column Sephadex™ G-25 using PBS as the buffer. The iodination protocol yielded labeled IgG chimeric antibody with a specific activity of 0.05 to 0.2 µCi/µg.

25

Female athymic mice (nu/nu) on a CD1 background were obtained from Charles River at approximately 4 weeks of age. Nine days later, mice were inoculated subcutaneously (0.1 mL/mouse) with LS174T cells (1 x 10⁶ cells/animal).

30

Athymic mice bearing carcinomas 70 to 400 mg in weight, approximately 12 to 13 days after inoculation of the cells were given injections intravenously of from 0.5 to 2.0 µCi (10-50 µg protein) in PBS of the chimeric monoclonal antibodies, which had been iodinated as

described above. Groups of five mice were sacrificed at varying times by exsanguination, the carcinoma and normal tissues were excised and weighed, and the cpm were measured in a gamma counter. The cpm/mg of each tissue was then determined and compared to that found in the carcinoma.

The results for CH44-1 are shown in Tables 1-2, and Figures 39A, 39B, and 39C. The results for CH84-1 are shown in Tables 3-4, and Figures 40A and 40B.

Percent Injected Dose Per Gram of ^{125}I -Labeled Antibody

Table 1

5	Tissue	CH44-1			
		0.75 Hour	23.5 Hours	49.5 Hours	122 Hours
	blood, total	29.70	15.84	8.09	7.31
10	Liver	8.13	4.13	2.19	1.96
	Spleen	6.19	3.39	2.12	1.36
	Kidney	4.35	2.80	1.52	1.33
	tumor	3.31	25.95	28.83	44.16
15	lung	7.34	5.39	2.90	2.36
	tumor, wt	0.18	0.12	0.09	0.11

20 As shown in Table 1, at approximately 122 hours post-injection, the percent injected dose to tumor for CH44-1 was 44.16 percent. CH44-1 was, therefore, efficient in targeting the human tumor in-situ. This demonstrates that the chimeric monoclonal antibodies of
25 the present invention were efficient for *in vivo* carcinoma targeting and thus are useful for *in vivo* treatment of cancer.

30

Percent Injected Dose Per Organ of ^{125}I -Labeled Antibody

Table 2

Tissue	CH44-1			
	0.75 Hour	23.5 Hours	49.5 Hours	122 Hours
blood, total	47.72	23.03	13.29	12.01
Liver	10.97	5.20	3.20	2.69
Spleen	1.09	0.48	0.25	0.22
Kidney	1.25	0.72	0.42	0.40
tumor	0.57	3.08	2.82	4.55
lung	1.20	0.87	0.57	0.37
GI tract	6.64	4.78	3.96	2.83
carcass	43.17	49.68	35.35	29.95
whole body retention	91.30	76.34	53.28	46.20

As shown in Table 2, at 122 hours post-injection, the percent of injected dose tumor for CH44-1 was 4.55 percent. CH84-1 was, therefore efficient in targeting the human tumor in-situ. This demonstrates that the chimeric monoclonal antibodies of the present invention were efficient for *in vivo* carcinoma targeting and thus were useful in *in vivo* treatment of cancer.

Percent Injected Dose Per Gram of ^{125}I -Labeled Antibody

Table 3

Tissue	CH84-1			
	1 Hour	23 Hours	47 Hours	118-119 Hours
blood	30.68	15.65	6.74	6.49
Liver	12.55	4.26	2.35	1.57
Spleen	10.93	3.35	2.56	1.70
Kidney	5.59	2.51	1.53	1.55
tumor	4.06	20.52	17.58	30.27
lung	10.77	4.80	2.58	2.24
tumor, wt.	0.15	0.22	0.20	0.24

As shown in Table 3, at approximately 118 hours post-injection, the percent of injected dose to tumor for CH84-1 was 30.27 percent. CH84-1 was, therefore, efficient in targeting the human tumor in-situ. This demonstrates that the chimeric monoclonal antibodies of the present invention were efficient for *in vivo* carcinoma targeting and thus were useful in *in vivo* treatment of cancer.

Percent Injected Dose Per Organ of ^{125}I -Labeled Antibody

Table 4

Tissue	CH84-1			
	1 Hour	23 Hours	47 Hours	118-119 Hours
blood, total	45.98	22.11	10.08	9.37
Liver	13.64	5.34	3.13	1.94
Spleen	1.35	0.49	0.32	0.16
Kidney	1.39	0.62	0.38	0.38
tumor	0.59	4.33	3.63	7.02
lung	1.77	0.69	0.42	0.31
GI tract	7.38	4.92	3.41	2.32
carcass	44.83	52.19	30.32	24.06
whole body retention	93.58	81.00	47.14	45.48

As shown in Table 4, at approximately 118 post-injection, the percent of injected dose to tumor for CH84-1 was 7.02 percent. CH84-1 was, therefore, efficient in targeting the human tumor in-situ. This demonstrates that the chimeric monoclonal antibodies of the present invention were efficient for *in vivo* carcinoma targeting and thus were useful in *in vivo* treatment of cancer.

Deposit of Cell lines Producing Chimeric Antibodies

Eleven illustrative cell lines secreting chimeric antibodies, all having a kappa light chains, made by the above examples were deposited at the American Type Culture Collection (ATCC) on October 19, 1988. Specifically, the following cell lines have been deposited: (1) CH44-1: a cell line having CC49 V_H, CC49 V_L, and constant region of IgG₁ (ATCC No. HB 9884); (2) CH44-2: a cell line having CC49 V_H, CC49 V_L, and constant region of IgG₂ (ATCC No. HB 9880); (3) CH44-4: a cell line having CC49 V_H, CC49 V_L, and constant region of IgG₄ (ATCC No. 9877); (4) CH88-1: a cell line having V_H, CC83 V_L, and constant region of IgG₁ (ATCC No. 9882); (5) CH88-2: a cell line having CC83 V_H, CC83 V_L, and constant region of IgG₂ (ATCC No. 9881); (6) CH88-3: a cell line having CC83 V_H, CC83 V_L, and constant region of IgG₃ (ATCC No. 9876); (7) CH88-4: a cell line having CC83 V_H, CC83 V_L, and constant region of IgG₄ (ATCC No. 9874); (8) CH84-1: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₁ (ATCC No. 9883); (9) CH84-2: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₂ (ATCC No. 9879); (10) CH84-3: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₃ (ATCC No. 9878); and (11) CH84-4: a cell line having CC83 V_H, CC49 V_L, and constant region of IgG₄ (ATCC No. 9875).

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiments are intended as a single illustration of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, while this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled

-131-

in the art that various changes and modifications could be made therein without departing from the spirit and scope of the appended claims.

5

10

15

20

25

30

1. An antibody or antibody fragment comprising:

5 a variable region having a heavy chain (V_H), said V_H being encoded by a DNA sequence effectively homologous to the V_H TAG germline gene (V_H TAG),

10 wherein the variable region binds to TAG72 at least 25 percent greater than the variable region of B72.3 binds to TAG72, with the binding affinities of the antibody and B72.3 being measured by the same technique.

15 2. An antibody or antibody fragment of Claim 1, wherein the V_H is further encoded by an animal D gene segment and an animal J gene segment.

20 3. The antibody or antibody fragment according to Claim 2, wherein the heavy chain D gene segment is encoded by a gene selected from murine and human D gene segments, and the J gene segment is encoded by a gene selected from murine and human J gene segments.

25 4. The antibody or antibody fragment of Claim 1, wherein the variable region further has a light chain (V_L) encoded by an animal V gene segment.

-133-

5. The antibody or antibody fragment of Claim 4, wherein the V_L is further encoded by an J gene segment.

5 6. The antibody or antibody fragment according to Claim 5, wherein the light chain J segment is encoded by a gene selected from murine and human J gene segments.

10 7. The antibody or antibody fragment of Claim 1, wherein the variable region is derived from the variable regions of CC46, CC49, CC83 or CC92.

15 8. The antibody or antibody fragment of Claim 1, wherein the variable region comprises (1) complementarity diversity regions (CDR) being encoded by a gene derived from the $V_{H\alpha}TAG$, and (2) framework regions, adjacent to the CDR regions, derived from human genes.

20 9. The antibody or antibody fragment of Claim 1, further comprising a constant region having at least a portion of a human light chain (C_L) and a human heavy chain (C_H).

25 10. The antibody or antibody fragment of Claim 9, wherein the C_H is IgG1-4, IgM, IgA, IgD or IgE.

11. The antibody or antibody fragment of Claim 9, wherein C_L is kappa or lambda.

30 12. The antibody or antibody fragment of Claim 1, wherein it is produced by a cell line from CH44-1, CH44-2, CH44-4, CH88-1, CH88-2, CH88-3, CH88-4, CH84-1, CH84-2, CH84-3 or CH84-4.

-134-

13. An antibody or antibody fragment conjugate comprising antibody or antibody fragment of any one of Claims 1 through 12 conjugated to an imaging marker.

14. The antibody or antibody fragment
5 conjugate of Claim 13, wherein the imaging marker is ^{125}I , ^{131}I , ^{123}I , ^{111}In , ^{105}Rh , ^{153}Sm , ^{67}Cu , ^{67}Ga , ^{166}Ho , ^{177}Lu , ^{186}Re , ^{188}Re or $^{99\text{m}}\text{Tc}$.

15. A antibody or antibody fragment conjugate
10 comprising the antibody or antibody fragment of any one of Claims 1 through 12 conjugated to a therapeutic agent.

16. The antibody or antibody fragment
15 conjugate of Claim 15, wherein the therapeutic agent is a radionuclide, drug or biological response modifier, toxin or another antibody.

17. The antibody or antibody fragment
20 conjugate of Claim 16, wherein the radionuclide is ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi , ^{211}At , ^{67}Ga , ^{125}I , ^{186}Re , ^{188}Re , ^{177}Lu , $^{99\text{m}}\text{Tc}$, ^{153}Sm , ^{123}I or ^{111}In .

18. The antibody or antibody fragment
25 conjugate of Claim 16, wherein the drug or biological response modifier is methotrexate, adriamycin or lymphokine.

19. A DNA sequence encoding at least a portion
30 of an antibody heavy chain,

said sequence comprising a DNA sequence segment being effectively homologous to the $\text{V}_\text{H}\alpha\text{TAG}$ germline gene ($\text{V}_\text{H}\alpha\text{TAG}$),

-135-

wherein the DNA sequence segment encodes at least a portion of a heavy chain variable region (V_H).

20. The DNA sequence of Claim 19, wherein the sequence further comprises a DNA sequence segment animal
5 D genes and a DNA sequence segment of animal J genes.

21. The DNA sequence of Claim 20, wherein the DNA sequence encoding the V_H is derived from sequences encoding the V_H regions of CC46, CC49, CC83, and CC92.

10 22. The DNA sequence of Claim 20, wherein the sequence encodes for a V_H having (1) at least one complementarity diversity region (CDR) encoded by a gene effectively homologous to the V_HαTAG, and (2) framework
15 regions, adjacent to the CDR regions, encoded by a human gene.

23. The DNA sequence of Claim 19, further comprising sequence segment encoding for at least a
20 portion of a human heavy chain constant region (C_H).

24. The DNA sequence of Claim 23, wherein the sequence segment encodes for at least a portion of a C_H gene encoding IgG₁₋₄, IgM, IgA, IgD or IgE.

25 25. A DNA sequence comprising:

(A) a sequence segment encoding for an antibody or antibody fragment heavy chain, said sequence segment
30 having

(1) a sequence subsegment being effectively homologous to the V_HαTAG germline gene (V_HαTAG), wherein the DNA sequence segment encodes at least a portion of a V_H, and

-136-

(2) a sequence subsegment encoding for at least a portion of a C_H ; and

(B) a sequence segment encoding for an antibody or antibody fragment light chain, said sequence segment
5 having

(1) a sequence subsegment encoding for at least a portion of an animal light chain variable region (V_L), and

10 (2) a sequence subsegment encoding for at least a portion of a human light chain constant region (C_L),

15 wherein the antibody or antibody fragment encoded by the DNA sequence binds to TAG72 at least 25 percent greater than the variable region of B72.3 binds to TAG72, with the binding affinities of the antibody and B72.3 being measured by the same technique.

20 26. The DNA sequence of Claim 25, wherein the sequence segment encoding for at least a portion of a C_H gene encoding IgG1-4, IgM, IgA, IgD or IgE.

25 27. The DNA sequence of Claim 25, wherein the sequence segment encoding for at least a portion of a C_L gene encoding kappa or lambda.

30 28. A biologically functional expression vehicle containing the DNA sequence of any one of Claims 19 through 27.

29. A cell transformed with the biologically functional expression vehicle of Claim 28.

-137-

30. A cell of Claim 29, which comprises the characteristics of a cell of CH44-1, CH44-2, CH44-4, CH88-1, CH88-2, CH88-3, CH88-4, CH84-1, CH84-2, CH84-3 or CH84-4.

5 31. A composition comprising the antibody or antibody fragment of any one of Claims 1 through 12, in a pharmaceutically acceptable, non-toxic, sterile carrier.

10 32. A composition comprising the antibody or antibody fragment conjugate of any one of Claims 13 through 14, in a pharmaceutically acceptable, non-toxic, sterile carrier.

15 33. A composition comprising the antibody or antibody fragment conjugate of any one of Claims 15 through 18, in a pharmaceutically acceptable, non-toxic, sterile carrier.

20 34. A method for *in vivo* diagnosis of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 31 or 32 for the *in situ* detection of carcinoma lesions.

25 35. A method for *in vivo* therapy of cancer which comprises administering to an animal a pharmaceutically effective amount of the composition of Claim 31 or 33.

30 36. The method of Claim 34 or 35, wherein the animal is a human.

-138-

37. A method for intraoperative therapy which comprises:

(a) administering to an animal a pharmaceutically effective amount of the composition
5 Claim 31 or 32, whereby the tumors are localized, and

(b) excising of the localized tumors.

38. A process for preparing an antibody or
10 antibody fragment which comprises contacting:
a variable region having a heavy chain (V_H),
said V_H being encoded by a DNA sequence effectively
homologous to the V_H TAG germline gene (V_H TAG),
with a variable region having a light chain
15 (V_L), said V_L being encoded by an animal V gene segment
and an animal J gene segment,
to form a variable region of the antibody or
antibody fragment,
wherein the variable region binds to TAG72 at
20 least 25 percent greater than the variable region of
B72.3 binds to TAG72, with the binding affinities of the
antibody and B72.3 being measured by the same technique.

39. The process of Claim 38, wherein variable
25 region V_H further comprises:
being encoded by an animal D gene segment and
an animal J gene segment.

40. The process of Claim 39, wherein the light
30 chain J segment is encoded by a gene selected from
murine and human J gene segments.

41. The process of Claim 39, wherein the heavy
chain D gene segment is encoded by a gene selected from
murine and human D gene segments, and the J gene segment

is encoded by a gene selected from murine and human J gene segments.

42. The process of Claim 38, wherein the variable region is derived from the variable regions of
5 CC46, CC49, CC83 or CC92.

43. The process of Claim 38, wherein the variable region comprises (1) complementarity diversity regions (CDR) being encoded by a gene derived from the
10 V_HαTAG, and (2) framework regions, adjacent to the CDR regions, derived from human genes.

44. The process of Claim 38, wherein the antibody or antibody fragment further comprises a
15 constant region having at least a portion of a human light chain (C_L) and a human heavy chain (C_H).

45. The process of Claim 44, wherein the C_H is IgG1-4, IgM, IgA, IgD or IgE.
20

46. The process of Claim 44, wherein C_L is kappa or lambda.

47. The process of Claim 38, wherein the
25 antibody or antibody fragment is produced by a cell line from CH44-1, CH44-2, CH44-4, CH88-1, CH88-2, CH88-3, CH88-4, CH84-1, CH84-2, CH84-3 or CH84-4.

48. A process for preparing an antibody or
30 antibody fragment conjugate which comprises contacting: an antibody or antibody fragment, as defined in any one of Claims 1 through 12, with an imaging marker or therapeutic agent.

-140-

49. The process of Claim 48, wherein the imaging marker is ^{125}I , ^{131}I , ^{123}I , ^{111}In , ^{105}Rh , ^{153}Sm , ^{67}Cu , ^{67}Ga , ^{166}Ho , ^{177}Lu , ^{186}Re , ^{188}Re or $^{99\text{m}}\text{Tc}$.

50. The process of Claim 48, wherein the
5 therapeutic agent is a radionuclide, drug or biological response modifier, toxin or another antibody.

51. The process of Claim 50, wherein the radionuclide is ^{131}I , ^{90}Y , ^{105}Rh , ^{47}Sc , ^{67}Cu , ^{212}Bi ,
10 ^{211}At , ^{67}Ga , ^{125}I , ^{186}Re , ^{188}Re , ^{177}Lu , $^{99\text{m}}\text{Tc}$, ^{153}Sm , ^{123}I or ^{111}In .

52. The process of Claim 50, wherein the drug
or biological response modifier is methotrexate,
15 adriamycin or lymphokine.

53. A process for preparing a recombinant expression vehicle which comprises:

inserting a DNA sequence, said sequence
20 comprising a DNA sequence segment being effectively homologous to the $\text{V}_\text{H}\alpha\text{TAg}$ germline gene ($\text{V}_\text{H}\alpha\text{TAg}$),
wherein the DNA sequence segment encodes at least a portion of a heavy chain variable region (V_H),
into an expression vehicle.
25

54. The process of Claim 53, wherein the V_H sequence further comprises a DNA sequence segment animal
D genes segment and a DNA sequence segment of animal J
genes.
30

55. The process of Claim 54, wherein the DNA sequence encoding the V_H is derived from sequences encoding the V_H regions of CC46, CC49, CC83, and CC92.

56. The process of Claim 53, further comprising the DNA sequence segment encoding for at least a portion of a human heavy chain constant region (C_H).

57. The process of Claim 56, wherein the DNA
5 sequence segment encodes for at least a portion of a C_H gene encoding IgG1-4, IgM, IgA, IgD or IgE.

58. The process of Claim 53, wherein about 10
10 μ g of expression vehicle is contacted with about 10 μ g of the DNA sequence segment with 10 units of enzyme in 10 μ L of buffer solution.

59. The process of Claim 53, wherein a
15 digestion is carried out at temperatures ranging from 37° to 65°C, at a pH of 7-9, for 1 to 18 hours.

60. A process for preparing a transformed host
which comprises inserting an expression vehicle, as
defined in Claim 28, into a suitable host.

20 61. The process of Claim 60, wherein the expression vehicle is a plasmid.

62. The process of Claim 61, wherein 10-30 μ g
25 of plasmid were linearized in the presence of up to 1×10^7 host cells, followed by electroporating the host cells at 200 volts and 960 μ Farads.

30

1 / 4 8

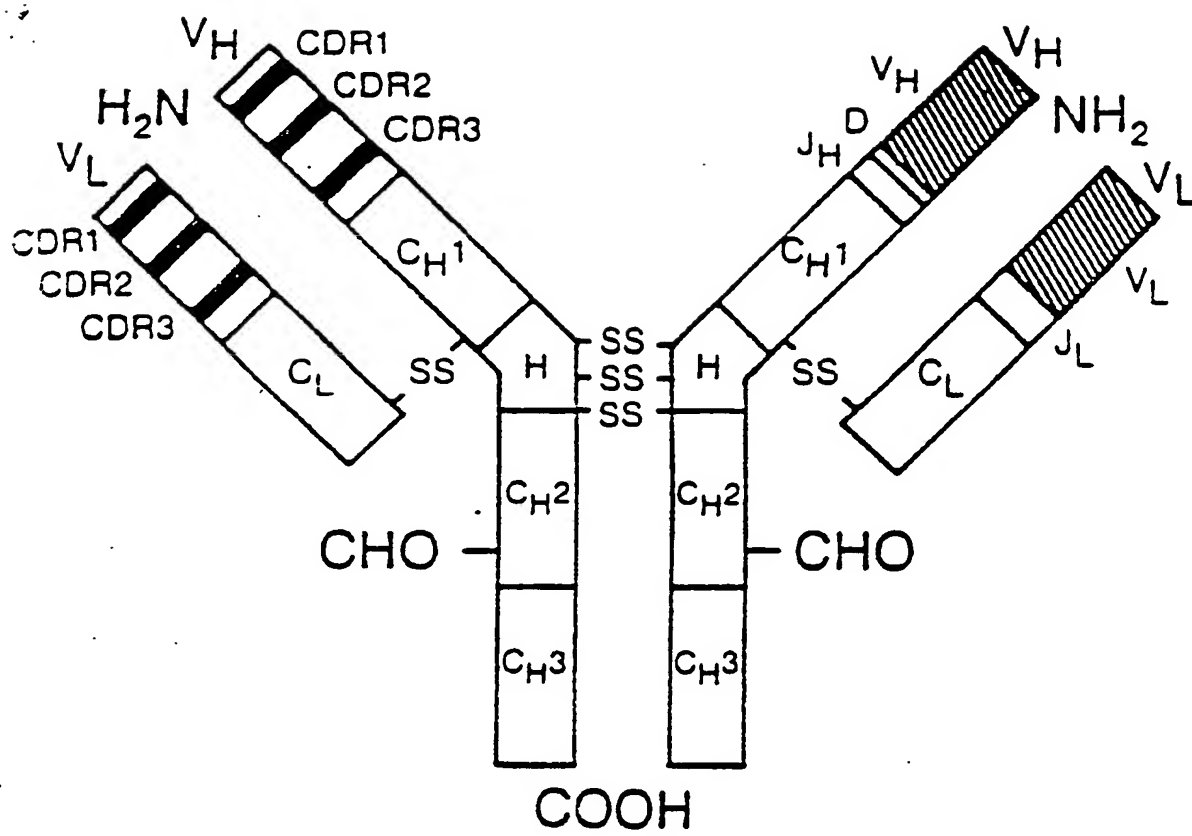


Figure 1

CCTTCTCTTCCTCCACCACCAATCCACATTGTGAATCAAC
.....
.....

ATCTTTAACNTATCACAGAGTGGAGCAACAGGATTCAGGGCAAAAATATGCTGAGAGATTATATCCCTGTTCGTTACAAACCAAGCATC
T.....

[illegible][illegible]

GCAGTAAATATACAGAGATGTTCATATACCA"IAAAAACAATATATGATCAGTGTCTTCTCCGCTATCCCCTGGACACACTGACTCTAACC

[illegible]

Figure 2

3 / 4 8

(intron)																							
VH μ ITAG	TCT	AA	GTGG	AGTC	AGGG	CCCTG	AGG	GTG	ACAG	TTC	GGC	TTC	ACAC	AG	GT	GTC	CAC	TCC	CAG	GTT	CAG	197	
CC46		
CC49		
CC83		
CC92		
VH μ ITAG	CTG	CAG	CAG	TCT	GAC	GCT	GAG	TTG	GTG	AAA	CCT	GGG	GCT	TCA	GTG	AAG	ATA	TCC	TGC	AAG	GCT	260	
CC46	..AGT		
CC49	T..T		
CC83	T..T		
CC92		
CDR 1																							
VH μ ITAG	TCT	GGC	TAC	ACC	TTC	ACT	GAC	CAT	GCT	ATT	CAC	TGG	GTG	AAG	CAG	AAG	CCT	GAA	CAG	GGC	CTG	323	
CC46		
CC49AAC		
CC83		
CC92A		
CDR 2																							
VH μ ITAG	GAA	TGG	ATT	GGA	TAT	ATT	TC	T	CCC	GGA	AAT	GGT	GAT	ATT	AAG	TAC	AAT	GAG	AAG	TTC	AAG	386	
CC46		
CC49ATA		
CC83A		
CC92A		
VH μ ITAG	AAG	GCC	ACA	CTG	ACT	GCA	GAC	AAA	TCC	TCC	AGC	AGC	ACT	GCC	TAC	ATG	CAG	CTC	AAC	AGC	CTG	ACA	409
CC46T	
CC49G	
CC83TA	
CC92CAT	

Figure 2 continued

4 / 4 8

V_HμTAG 48 J
 CC46 TCT GAG GAT TCT GCA GTG TAT TTC TGT AAA AGA CACAGTGTGTGTAACACACATCCCTGAGTGTGTGTCAGAAATCCCTG
 CC49CCG G.C GGC TAC GGG GTT GCT TTT TGG GGC CAA GGG
 CC83CC. ... TCC CTG AAT ATG GCC TAC TGG GGT CAA GGA
 CC92CG. ... TCC TTC TAC GGC AAC --- TGG GGC CAA GGC
C. ... TCT CTA TCC GGG GAC TCC TGG GGC CAG GGC
 ----- CDR 3 -----

V_HμTAG 568
 CC46 GGGGAGCAGAAAGATACACTGGGGACTGAGAGACAGAGAAATTAATCCCTTACACCTTGCCTCAGAAATCGTAATTTTGAATGCCCTAT
 CC49 ACT CTG GTC ACT GTC TCT GCA
 CC83 ACC TCA GTC ACC GTC TCC TCA
 CC92 ACC ACC CTC ACA GTC TCC TCA
 ACC ACT CTC ACA GTC TCC TCA

V_HμTAG 611
 TTAATTCATCTTGCTCAGACACCTATATTCCTTTTGTAGCTT

Figure 2 continued

5 / 4 8

V_HμTAG
 CC46
 CC49
 CC83
 CC92

-19	Met	Glu	Trp	Ser	Trp	Val	Phe	Leu	Phe	Phe	Leu	Ser	Val	Thr	Thr	Gly	Val	His	Ser	Gln	Val
	Phe

V_HμTAG
 CC46
 CC49
 CC83
 CC92

10	Gln	Leu	Gln	Gln	Ser	Asp	Ala	Glu	Leu	Val	Lys	Pro	Gly	Ala	Ser	Val	Lys	Ile	Ser	Cys	Lys

V_HμTAG
 CC46
 CC49
 CC83
 CC92

30	Ala	Ser	Gly	Tyr	Thr	Phe	Thr	Asp	His	Ala	Ile	His	Trp	Val	Lys	Gln	Lys	Pro	Glu	Gln	Gly

V_HμTAG
 CC46
 CC49
 CC83
 CC92

50	Leu	Glu	Trp	Ile	Gly	Tyr	Ile	Ser	Pro	Gly	Asn	Gly	Asp	Ile	Lys	Tyr	Asn	Glu	Lys	Phe	Lys

V_HμTAG
 CC46
 CC49
 CC83
 CC92

70	Gly	Lys	Ala	Thr	Leu	Thr	Ala	Asp	Lys	Ser	Ser	Ser	Thr	Ala	Tyr	Met	Gln	Leu	Asn	Ser	Leu

Figure 3

Figure 4a

(NUCLEOTIDE SEQUENCE OF CC-49 V₁)

1 CC ATC CAC CAC TCT CAC ACA CAC TGC CCA GGC ATT TGC TTT TGT ATT TGC 47
 48 TGG CTG CTT TGC ATA GAC CCC TCC AGC CTA ACC CAG CAG GGC CTG TCG ATT AGA ATT 95
 96 TAT AAA CCA CCA TGA TGA ACT GAG CAG CAT CAG ACA CAG CTA CAG GGC CTG TCG ATT AGA ATT 143
 144 ATG GAT TCA CAG GCC CAG GGT CTT ATG TTA CTA CTA CTA CTA CTA CTA CTA CTA CTA CTA 191
 192 GGT GAG AAA TTT AAA ACT TTT AAA ACT TTT AAA ACT TTT AAA ACT TTT AAA ACT TTT AAA 239
 240 TAA GAA ATT TAT ACT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT 287
 288 CTC TGA CAA TAT GAC ATT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT 335
 336 TAA TAA TCT ATT TGT GAT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT 383
 384 GGT ACC TGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT 431
 432 GTG TCA GTT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT GGT 479
 480 CTT TTA TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT 527
 528 AAA CCA GGG CAG TCT CCT AAA CAG CTG ATT TAC TAC TAC TAC TAC TAC TAC TAC TAC TAC 575
 576 GAA TCT GGG GTC CCT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT GAT 623
 624 TTC ACT CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC CTC 671
 672 TAC TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT 719
 720 AAG CTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG GTG 767
 768 TAA GAC ACA GGT TTT CAT GTT AGG AGT T

The underlined segments show the portions verified by mRNA sequencing.

Figure 4b

(AMINO ACID SEQUENCE OF CC-49 VL)

VAL	SER	GLY	MET	ASP	SER	GLN	ALA	GLN	VAL	LEU	MET	LEU	LEU	LEU	LEU	TRP
<u>PRO</u>	<u>VAL</u>	<u>SER</u>	<u>VAL</u>	<u>GLY</u>	<u>GLY</u>	// <u>ASP</u>	<u>ILE</u>	<u>VAL</u>	<u>MET</u>	<u>SER</u>	<u>GLN</u>	<u>SER</u>	<u>PRO</u>	<u>SER</u>	<u>SER</u>	<u>LEU</u>
LEU	LEU	TYR	SER	<u>GLY</u>	<u>GLU</u>	<u>LYS</u>	<u>VAL</u>	<u>THR</u>	<u>LEU</u>	<u>SER</u>	<u>CYS</u>	<u>LYS</u>	<u>SER</u>	<u>SER</u>	<u>GLN</u>	<u>SER</u>
PRO	GLY	GLN	SER	GLY	ASN	GLN	LYS	ASN	TYR	LEU	ALA	TRP	TYR	GLN	GLN	LYS
GLY	VAL	PRO	SER	PRO	LYS	LEU	LEU	ILE	TYR	TRP	ALA	SER	ALA	ARG	GLU	SER
SER	ILE	SER	ASP	ARG	PHE	THR	GLY	SER	GLY	SER	GLY	THR	ASP	PHE	THR	LEU
TYR	TYR	SER	TYR	VAL	LYS	THR	GLU	ASP	LEU	ALA	VAL	TYR	TYR	CYS	GLN	GLN
				PRO/	LEU	THR	PHE	GLY	ALA	GLY	THR	LYS	LEU	VAL	LEU	LYS

8 / 4 8

"/" indicates the end of the V segment and the beginning of the J5 segment.

"/" Indicates the end of the leader sequence and the beginning of the mature protein.

The amino acid sequence determination for the first 24 amino acids of the mature CC49 VL are underlined.

9 / 4 8

Figure 5a

(NUCLEOTIDE SEQUENCE OF CC-83 V₁)

1	CC	AG	GG	CAG	CAC	AGC	TGC	CCA	TGA	TTT	ATA	AAC	CAG	GTC	TTT	GCA	48
49	GTG	AGA	TCT	CAA	ATA	CAT	CAG	ACC	AGC	ATG	GGC	ATC	AAG	ATG	GAG	ACA	96
97	CAT	TCT	CAG	GTC	TTT	GTA	TAC	AIG	TIG	CIG	TGG	TIG	TCT	GGT	GAG	A	142
143	CAT	TTA	AAA	GTA	TTA	TAA	AAT	CTT	AAA	AGT	AAT	CTA	TTT	AAA	TAG	CTT	190
191	TTT	CCT	ATA	GGA	AGC	CAA	TAT	TAG	GCA	GAC	AAT	GCC	ATT	AGA	TAA	GAC	238
239	ATT	TIG	GAT	TCT	AAC	ATT	TGT	ATC	TIG	AAG	TCT	TTA	TAT	GTG	TGA	GTT	286
287	TAT	ACA	CAT	TAT	CTG	TTT	CTG	TTT	GCA	GGT	GTT	GAA	GGA	GAC	ATT	GTG	334
335	ATG	ACC	CAG	TCT	CAC	AAA	TTC	ATG	TCC	GCA	TCA	GTG	GGA	GAC	AGG	GTC	382
383	AAC	ATC	ACC	TGC	AAG	GCC	AGT	CAG	TAT	GIG	GCT	ACT	GCT	GTA	GCC	TGG	430
431	TTT	CAG	CAT	AAA	CCA	GGT	CAG	TCT	CCT	AAA	CTA	CTG	ATT	TAC	GGG	GCA	478
479	TCC	ACC	CGG	CAC	ACT	GGA	GTC	CCI	GAT	CGC	TTC	ACA	GGC	AGT	GGA	TCT	526
527	GGG	ACA	GAT	TTC	ACT	CTC	ATC	ATT	AGC	AAT	GTG	CAG	TCT	GAG	GAC	TTG	574
575	GCA	GAT	TAT	TTG	TGT	CAG	CAT	TAT	AGC	GGC	TAT	CCA	TTC	ACG	TTC	GGC	622
623	TCG	GGG	ACA	AAG	TTG	GAA	ATA	AAA	CGT	AAG	TAG	ACT	TTT	GCT	CAT	TTA	670
671	CTT	GTG	ACG	TTT	TGG												

The underlined segments show the portions verified by mtRNA sequencing.

10 / 48

Figure 5b

(AMINO ACID SEQUENCE OF CC-83 V₁)

<u>MET</u>	GLY	ILE	LYS	MET	GLU	THR	ILE	SER	GLN	VAL	PIIE	VAL	TYR	MET	LEU	LEU
TRP	LEU	SER	GLY	VAL	GLU	GLY	//ASP	ILE	VAL	MET	TIIR	GLN	SER	IIIS	LYS	PIIE
<u>MET</u>	<u>SER</u>	<u>ALA</u>	<u>SER</u>	<u>VAL</u>	<u>GLY</u>	<u>ASP</u>	<u>ARG</u>	<u>VAL</u>	<u>ASN</u>	<u>ILE</u>	<u>TIIR</u>	<u>CYS</u>	<u>LYS</u>	<u>ALA</u>	<u>SER</u>	<u>GIN</u>
<u>TYR</u>	<u>VAL</u>	<u>ALA</u>	<u>TIIR</u>	<u>ALA</u>	<u>VAL</u>	<u>ALA</u>	TRP	PHE	GLN	IIIS	LYS	PRO	GLY	GLN	SER	PRO
<u>LYS</u>	<u>LEU</u>	<u>LEU</u>	<u>ILE</u>	<u>TYR</u>	<u>GLY</u>	<u>ALA</u>	SER	THR	ARG	IIIS	TIIR	GLY	VAL	PRO	ASP	ARG
PIIE	TIIR	GLY	SER	GLY	SER	GLY	TIIR	ASP	PHE	TIIR	LEU	ILE	ILE	SER	ASN	VAL
GLN	SER	GLU	ASP	LEU	ALA	ASP	TYR	LEU	CYS	GLN	IIIS	TYR	SER	GLY	TYR	PRO
PIIE	TIIR	PIIE	GLY	SER	GLY	THR	LYS	LEU	GLU	ILE	LYS					

"/" indicates the end of the V segment and the beginning of the J4 segment.

"/" indicates the end of the leader sequence and the beginning of the mature protein.

* ASN20 is not determined. It occurs in the consensus sequence ASN-X-SER/Thr where N-glycosylation is likely. The amino acid sequence determination for the first 51 amino acids of the mature CC83 V₁ are underlined.

11 / 48

CC-92 Light Chain Variable Region Sequence

GAGTCACAGATCCAGGTCCTTTGTATTCGTGTTTCTCTGGTTGTCTGGTGTGACGGAGA
CATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCA
T
CACCTGCAAGGCCAGTCAGGATGTGAGTAGTGCTGTAGGGTGGTTTCAACAGAAACCA
GG
ACAATCTCCTAAATTACTGATTTATTCGGCATCCTACCGGTATACTGGAGTCCCTGATCG
CTTCACTGGCAGTGGATCTCGGACGGATTTCACTTTCACCATCACCAGTGTGCAGGCTGA
AGACCTGGCAGTTTATTACTGTCAGCAACATTATAGTAGTCCGCTCACCTTCGGTGCTGG
GACCAAGCTGGAGCTGAAAC*

Figure 6a

12 / 48

```

Ser His Arg Ser Arg Ser Phe Val Phe Val Phe Leu Trp Leu Ser Gly Val Asp Gly
Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly Asp Arg Val
Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ser Ala Val Gly Trp Phe Gln Gln
Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly
Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Arg Thr Asp Phe Thr Phe Thr Ile Thr
Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln His Tyr Ser Ser Pro
Leu Thr Phe Gly Ala Gly Thr Lys Leu Gly Leu Lys

```

Figure 5b: Amino Acid Sequence of CC92 light chain variable region

Mouse Ig kappa germline J-C region, J1 to J5

AAGCTTTGCGCTACCCACTGCTCTGTTCTTTCAGTGAGGAGGGTTTTGTACAGCCAG
ACAGTGGAGTACTACCACTGTGGTGGACGTTCCGTGEGAGGCACCAAGCTEGAAATCAA
AC
GTAAGTAGAATCCAAAGTCTCTTCTCCGTTGTCTATGTCTGTGGCTTCTATGTCTAA
AATGATGTATAAAATCTTACTCTGAAACCAGATTCTGGCACTCTCCAAGGCAAAGATAC
A
GAGTAACTCCGTAAGCAAAGCTGGGAATAGGCTAGACATGTTCTCTEGAGAATGAATG
CC
AGTGTAAATAATTAACACAAGTGATAGTTTCAGAAATGCTCAAAGAAGCAGGGTAGCCT
GC
CCTAGACAAACCTTACTCGGTGCTCAGACCATGCTCAGTTTTTGTATGGGGGTTGAGTG
AAGGGACACCACTGTGTGTACACGTTCCGAGGGGGGACCAAGCTGGAAATAAAACGT
AAG
TAGTCTTCTCAACTCTTGTTCACTAAGTCTAACCTTGTTAAGTTGTTCTTTGTTGTGTGT
TTTTCTTAAGGAGATTTTCAGGGATTTAGCAAATTCATTCTCAGATCAGGTGTTAAGGAG
GGAAAACGTGCCCACAAGAGGTTGGAATGATTTTCAGGCTAAATTTTAGGCTTTCTAAA
C
CAAAGTAACTAACTAGGGGAAGAGGGATAATTGTCTACCTAGGGAGGGTTTTGTGGA
GG
TAAAGTAAAATAAATCACTGTAAATCACATTCAGTGATGGGACCAGACTGGAAATAA
AA
CCTAAGTACATTTTTGCTCAACTGCTTGTGAAGTTTTGGTCCCATTTGTCTTTGTATG
AGTTTGTGGTGTACATTAGATAAATGAACTATTCCTTGTAACCCAAAACCTTAAATAGAA
G
AGAACC AAAAATCTAGCTACTGTACAAGCTGAGCAAACAGACTGACCTCATGTCAGATT
T
GTGGGAGAAATGAGAAAGGAACAGTTTTTCTCTGAACTTAGCCTATCTAACTGGATCCG
C
TCAGGCAGGTTTTTGTAAAGGGGGCGCAGTGATATGAATCACTGTGATTCACGTTCCG
C
TCGGGGACAAAGTTGGAAATAAAACGTAAGTAGACTTTTGCTCATTTACTGTGACCT
T
TGGTTCTGTTTGGGTAACCTTGTGTGAATTTGTGACATTTTGGCTAAATGAGCCATTCCTG
GCAACCTGTGCATCAATAGAAGATCCCCCAGAAAAGAGTCAGTGTGAAAGCTGAGCGA
AA
AACTCGTCTTAGGCTTCTGAGACCAGTTTTGTAAGGGGAATGTAGAAGAAAGAGCTGG
GC
TTTTCTCTGAATTTGGCCCATCTAGTTGGACTGGCTTACAGGCGAGGTTTTGTAGAGA
GGGGCATGTCATAGTCCTCACTGTGGCTCACGTTCCGGTGCTGGGACCAAGCTGGAGCTG
A
AACGTAAGTACACTTTTCTCATCTTTTTTATGTGTAAGACACAGGTTTTCATGTTAGGA
GTTAAAGTCAGTTCAGAAAATCTTGAGAAAATGGAGAGGGCTCATTATCAGTTGACGT
GG
CATACAGTGTGAGATTTTCTGTTTATCAAGCTAGTGAGATTAGGGGCAAAAAGAGGCT
T
AGTTGAGAGGAAAGTAATTAATACTATGGTCACCATCCAAGAGATTGGATCEGAGAAT
AA
GCATGAGTAGTTATTGAGATCTGGGTCTGACTGCAG*

Figure 7

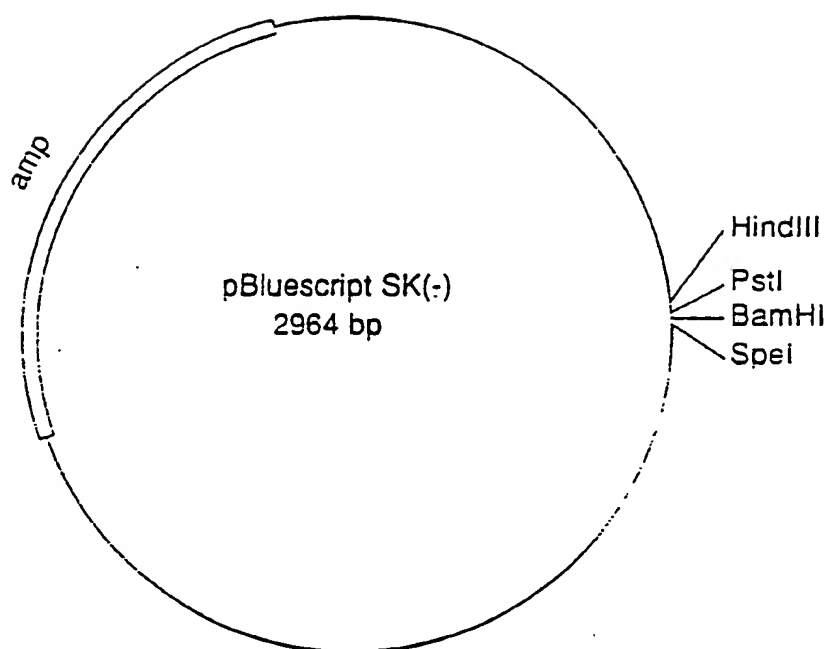


Figure 8

15 / 48

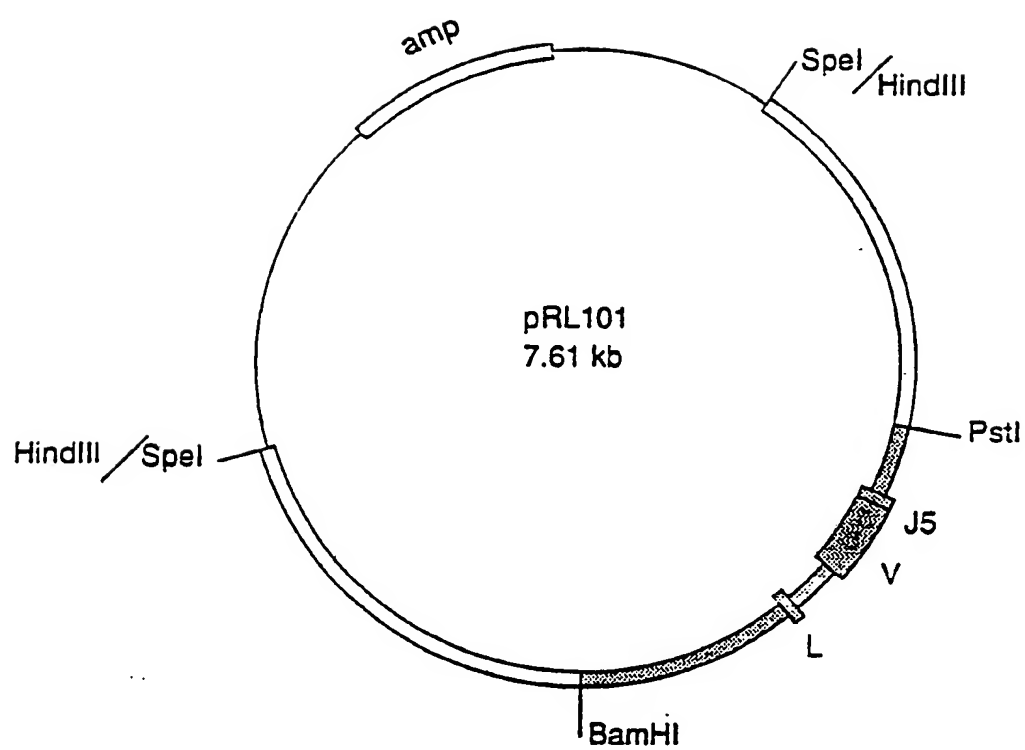
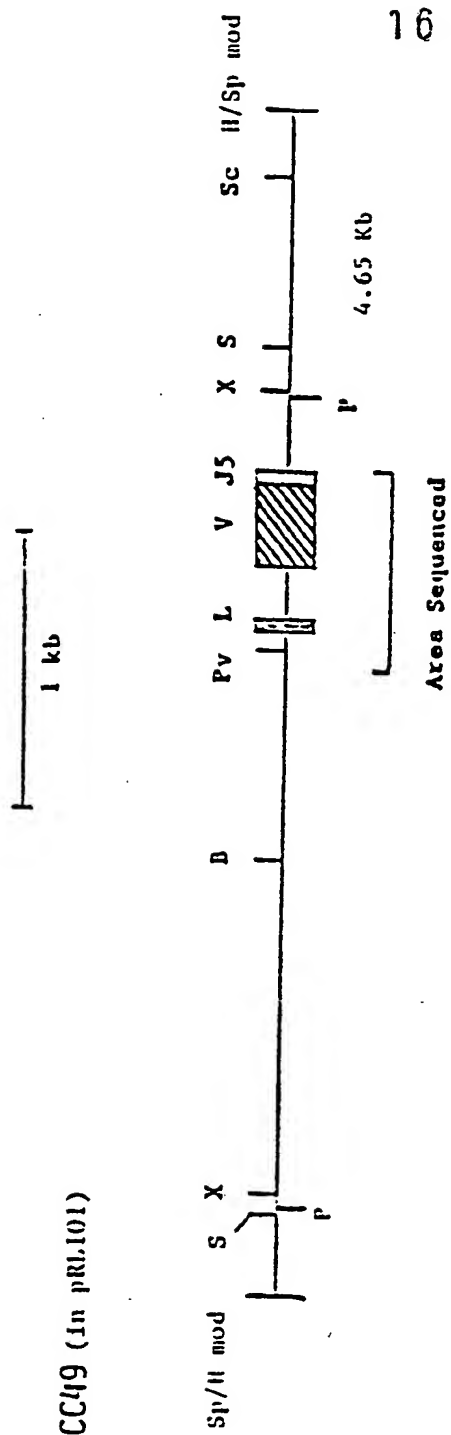


Figure 9

Figure 10



16 / 48

Key to
Restriction Enzymes:

- B - Bam HI
- Bgl - Bgl II
- C - Cla I
- E - Eco RI
- H - Hind III
- N - Nhe I
- P - Pst I
- Pv - Pvu II
- S - Sac I
- Sc - Sac II
- Sp - Spe I
- St - Stu I
- X - Xba I

17 / 48

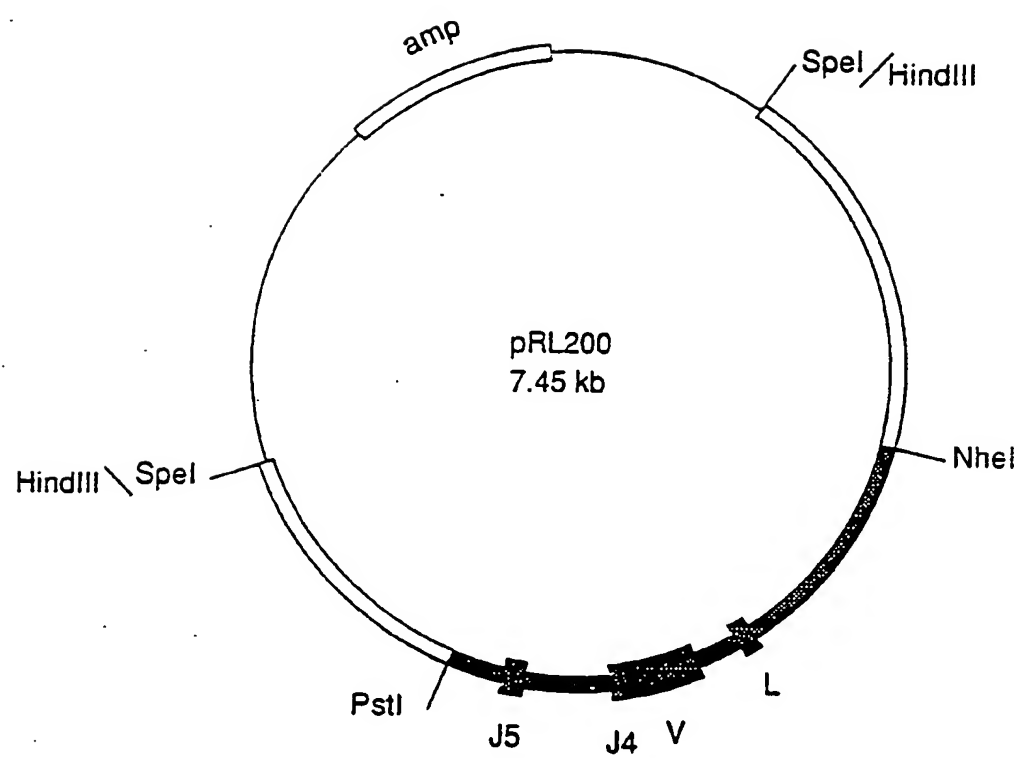
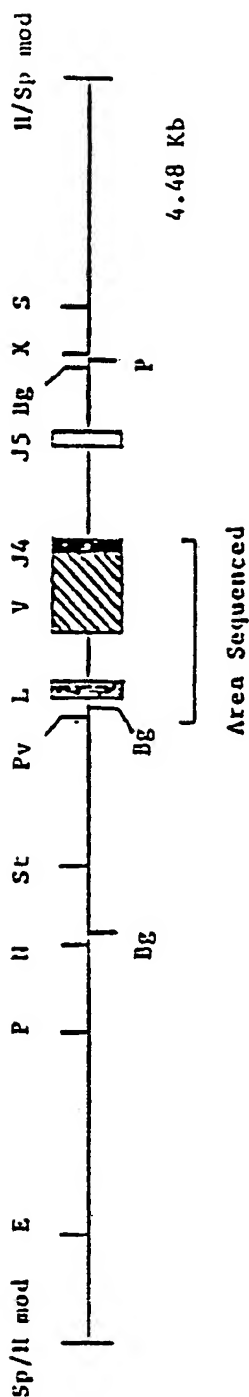


Figure 11

Figure 12

CC83 (in pRI.200)



Key to
Restriction Enzymes:

B - Bam HI
 Dg - Dgl II
 C - Cla I
 E - Eco RI
 H - Hind III
 N - Nhe I
 P - Pst I
 Pv - Pvu II
 S - Sac I
 Sc - Sac II
 Sp - Spe I
 St - Stu I
 X - Xba I

mod = half-filled in

Mouse Ig germline J-H genes from pNP9

GGATCCTGGCCAGCAATTGCCGCTAGGTCCCTCTCTTCTATGCTTTCTTTGTCCCTCACTG
GCCTCCATCTGAGATAATCCTGGAGCCCTAGCCAAGGATCATTTATTGTGAGGGGCTAA
TCATTGTTGTGACAATGTGCCTGGTTTGCTTACTGGGGCCAAGGGGACTCTGGTCACTGTC
TCTGCAGGTGAGTCTAACTTCTCCCATCTAAATGCATGTTGGGGGGAATCTGAGCCTT
CAGGACCAAGATTCTCTGCAAACGGGAATCAAGATTCAACCCCTTTGTCCCAAGTTGA
G
ACATGGGTCTGGGTCAAGGACTCTCTGCCTGCTGGTCTGTGGTGACATTAGAAGTGAAG
T
ATGATGAAGGATCTGCCAGAACTGAAGCTTGAAGTCTGAGGCAGAATCTGTCCAGGG
TC
TATCGGACTCTTGTGAGAATTAGGGGCTGACAGTTGATGGTGACAATTCAGGGTCAGT
G
ACTGTCAGGTTTCTCTGAGGTGAGGCTGGAATATAGGTCACCTTGAAGACTAAAGAGG
GG
TCCAGGGGCTTTTCTGCACAGGCAGGGAACAGAATGTGGAACAATGACTTGAATGGTT
GA
TTCTTGTGTGACACCAAGAATTGGCATAATGTCTGAGTTGCCCAAGGGTGATCTTAGCTA
GACTCTGGGGTTTTTGTGCGGTACAGAGGAAAAACCCACTATTGTGATTACTATGCTATG
GACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCTCAGGTAAGAATGGCCTCTCCAG
G
TCTTTATTTTAACTTTGTTATGGAGTTTTCTGAGCATTGCAGACTAATCTTGGATATT
TGCCCTGAGGGAGCCGCTGAGAGAAAGTTGGGAAATAAATCTGTCTAGGGATCTCAGA
GC
CTTTAGGACAGATTATCTCCACATCTTTGAAAACTAAGAATCTGTGTGATGGTGTGGT
GGAGTCCCTGGATGATGGGATAGGGACTTTGGAGGCTCATTTGAGGGAGATGCTAAAA
CA
ATCCTATGGCTGGAGGGATAGTTGGGGCTGTAGTTGGAGATTTTCAGTTTTTAGAATGA
A
GTATTAGCTGCAATACTTCAAGGACCACCTCTGTGACAACCATTTTATACAGTATCCAGG
CATAGGGACAAAAAGTGGAGTGGGGCACTTTCTTTAGATTTGTGAGGAATGTTCCACAC
T
AGATTGTTTAAACCTCATTGTTGGAAGGAGCTGTCTTAGTGATTGAGTCAAGGGAGA
A
AGGCATCTAGCCTCGGTCTCAAAAGGGTAGTTGCTGTCTAGAGAGGTCTGGTGGAGCCT
G
CAAAAGTCCAGCTTCAAAGGAACACAGAAGTATGTGTATGGAATATTAGAAGATGTT
GC
TTTTACTCTTAAGTTGGTTCTAGGAAAAATAGTTAAATACTGTGACTTTAAAATGTGAG
AGGGTTTTCAAGTACTCATTTTTTAAATGTCCAAAATTTTTGTCAATCAATTTGAGGTC
TTGTTTGTGTAGAACTGACATTACTTAAAGTTTAAACCGAGGAATGGGAGTGAGGCTCTC
T
CATACCCTATTGAGAACTGACTTTTAAACAATAATAAATTAAGTTTAAAATATTTTTAAAT
GAATTGAGCAATGTTGAGTTGAGTCAAGATGGCCGATCAGAACCAGGAACACCTGCAGC
AG
CTGGCAGGAAGCAGGTCATGTGGCAAGGCTATTTGGGGAAGGGAAAAATAAAACCACT
AGG
TAAACTTGTAGCTGTGGTTTGAAGAAGTGGTTTTGAAACACTCTGTCCAGCCCCACCAA
CCGAAAGTCCAGGCTGAGCAAAACACCACCTGGGTAATTTGCATTTCTAAAATAAGTTG
A
GGATTGAGCCGAAACTGGAGAGGTCCTCTTTTAACTTATTGAGTTCAACCTTTTAAATTT
AGCTTGAGTAGTTCTAGTTTCCCAAACCTTAAGTTTATCGACTTCTAAAATGTATTTAGA
ATTC
?

Figure 13

20 / 48

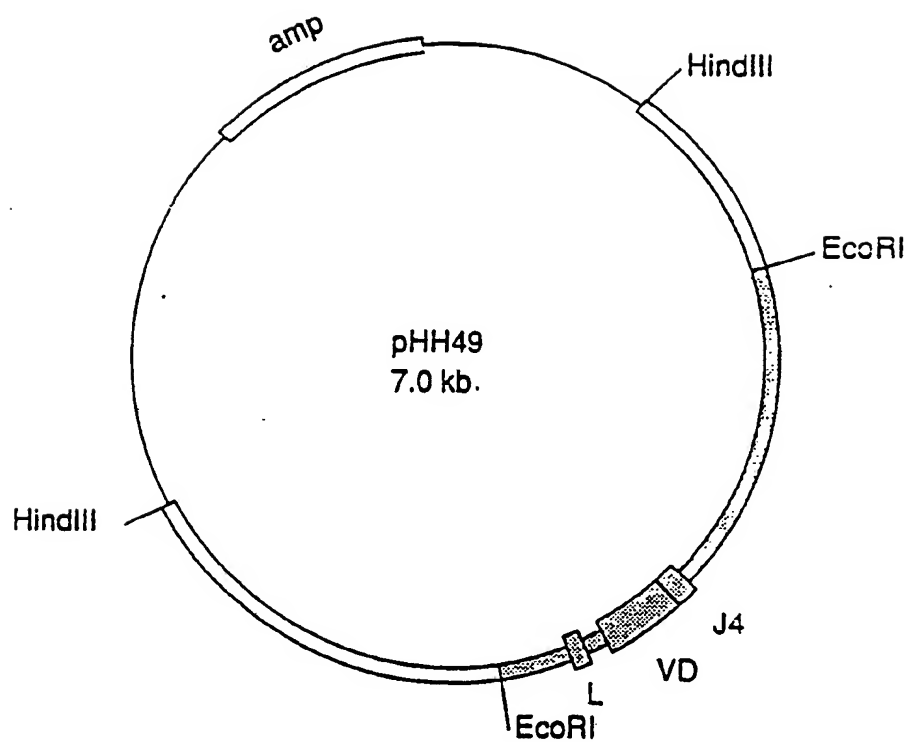


Figure 14

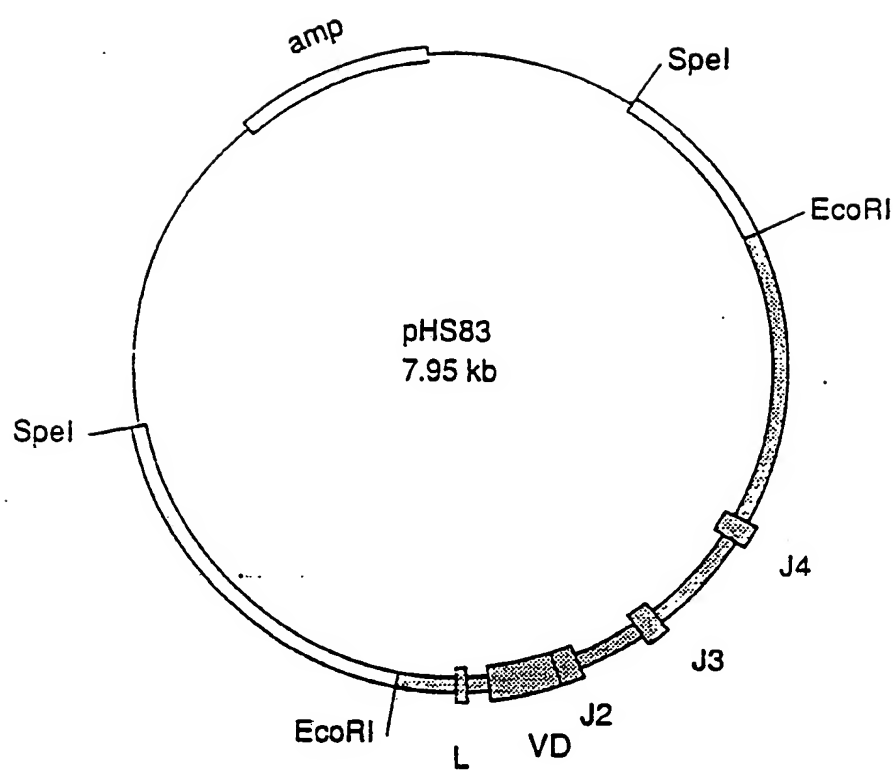


Figure 15

Figure 16

(NUCLEOTIDE SEQUENCE OF CC-49 V_{II})

239	TIA	ACA	IAT	CAC	AGA	GTG	GAG	CAA	CAG	AAT	CAG	GGC	AAA	AAT	ATG	CTG	286
287	AGA	GAT	TTT	TCC	CTG	TCG	TIA	CAA	CCA	AAG	CAT	CTG	TCT	AGA	ATT	CAT	334
335	AAA	AAC	TTT	ATG	GGA	TAC	ATT	TCC	TCA	GAG	AGG	AAT	AGG	ATT	TGG	ACC	382
383	TGA	GCA	TCC	TGC	TGC	CCG	AGC	CAT	GTG	ATG	ACA	GTT	CTT	CTC	CAG	TTG	430
431	AAC	TAG	GTC	CTT	ATC	TAA	GAA	ATG	CAC	TGC	TCA	TGA	TAT	GCA	AAT	CAC	478
479	CCG	AGT	CTA	TGG	CAG	TAA	ATA	CAG	AGA	TGT	TCA	TAC	CAT	AAA	AAC	AAT	526
527	ATG	TGA	TCA	GTG	TCT	TCT	CCG	CTA	TCC	CTG	GAC	ACA	CTG	ACT	CTA	ACC	574
575	ATG	GAA	TGG	AGC	TGG	GTC	TTT	CTC	TTC	TTC	CTG	TCA	GTA	ACT	ACA	GGTA	623
624	AGG	GGC	TCA	CCA	TTT	CCA	AAT	CTA	AAG	TGG	AGT	CAG	GGC	CTG	AGG	TGA	671
672	CAA	AGA	TGT	CCA	CTT	TGG	CTG	TCC	ACA	GGT	GTC	CAC	TCC	CAG	GTT	CAG	719
720	TTG	CAG	CAG	TCT	GAC	GCT	GAG	TTG	GTG	AAA	CCT	GGG	GCT	TCA	GTG	AAG	767
768	ATT	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACT	GAC	CAT	GCA	ATT	CAC	815
816	TGG	GTG	AAA	CAG	AAC	CCT	GAA	CAG	GGC	CTG	GAA	TGG	ATT	GGA	TAT	TTT	863
864	TCI	CCC	GGG	AAT	GAT	GAT	TTT	AAA	TAC	AAT	GAG	AGG	TTC	AAG	GGC	AAG	911
912	GCC	ACA	CTG	ACT	GCA	GAC	AAA	TCC	TCC	AGC	ACT	GCC	TAC	GTG	CAG	CTC	959
960	AAC	AGC	CTG	ACA	ICT	GAG	GAT	TCI	GCA	GTG	TAT	TTC	TGT	ACA	AGA	TCC	1007
1008	CTG	AAT	ATG	ATG	TAC	TGG	GGT	CAA	GGA	ACC	TCA	GTC	ACC	GTC	TCC	TCAG	1056

The underlined segments show the portions verified by mRNA sequencing.

(NUCLEOTIDE SEQUENCE OF CC-83 V_{II})

Figure 17

23 / 48

478	11A	ACA	TAT	CAC	AGA	GIG	GAG	CAA	CAG	AAT	CAG	GGC	AAA	AAT	ATG	CTG	525
526	AGA	GAT	TTA	TCC	CTG	TCG	TAA	CAA	CCA	AAG	CAT	CTG	TCT	AGA	ATT	CAT	573
574	AAA	AAC	TTT	ATG	GGA	TAC	TAC	ATT	TCC	TCA	GAG	AAT	AGG	ATT	TGG	ACC	621
622	TGA	GCA	TCC	TGC	TGC	CCG	AGC	CAT	GTG	ATG	ACA	GTT	CTT	CTC	CAG	TIG	669
670	AAC	TAG	GTC	CTT	ATC	TAA	GAA	ATG	CAC	TGC	TCA	TGA	TAT	GCA	AAT	CAC	717
718	CCG	AGT	CTA	TGG	CAG	TAA	ATA	CAG	AGA	TGT	TCA	TAC	CAT	AAA	AAC	AAT	765
766	ATA	TGA	TCA	GTG	TCT	TCT	CCG	CTA	TCC	CTG	GAC	ACA	CTG	ACT	CTA	ACC	813
814	ATG	GAA	TGG	AGC	TGG	GTC	TTT	CTC	TTC	TTC	CTG	TCA	GTA	ACT	ACA	GGTA	862
863	AGG	GGC	TCA	CCA	TTT	CCA	AAT	CTA	AAG	TGG	AGT	CAG	GGC	CTG	AGG	TGA	910
911	CAA	AGA	TAT	CCA	CTT	TGG	CTT	TCC	ACA	GGT	GTC	CAC	TCC	CAG	GTT	CAG	958
959	TTG	CAG	CAG	TCT	GAC	GCT	GAG	TTG	GTG	AAA	CCT	GGG	GCT	TCA	GTG	AAG	1006
1007	AAT	TCC	TGC	AAG	GCT	TCT	GGC	TAC	ACC	TTC	ACT	GAC	CAT	GCT	ATT	CAC	1054
1055	TGG	GIG	AAG	CAG	AAG	CCT	GAA	CAG	GGC	CTG	GAA	TGG	ATT	GGA	TAT	ATT	1102
1103	TCT	CCC	GGA	AAT	GAT	GAT	AAT	AAG	TAC	AAT	GAG	AAG	TTC	AAG	GGC	AAG	1150
1151	GCC	ACA	CTG	ACT	GCA	GAC	AAA	TCC	TCC	AGT	ACT	GCC	TAC	ATG	CAA	CTC	1198
1199	AAC	AGC	CTG	ACA	TCT	GAG	GAT	TCT	GCA	GTG	TAT	TTC	TGT	AGA	AGA	TCC	1246
1247	TTC	TAC	GGC	AAC	TGG	GGC	CAA	GGC	ACC	ACC	CTC	ACA	GTC	TCC	TCA	G	

the underlined segments show the portions verified by mRNA sequencing.

24 / 48

(AMINO ACID SEQUENCE OF CC-49 V_{II})

Figure 18

MEI	GLU	TRP	SER	TRP	VAL	PIIE	LEU	PIIE	PHE	LEU	SER	VAL	THIR	THIR
GLY	VAL	HIS	SER	//GIN	VAL	GIN	LEU	GIN	GIN	SER	ASP	ALA	GLU	LEU
VAL	LYS	PRO	GLY	ALA	SER	VAL	LYS	ILE	SER	CYS	LYS	ALA	SER	GLY
TYR	THIR	PIIE	THIR	ASP	HIS	ALA	ILE	HIS	TRP	VAL	LYS	GIN	ASN	PRO
GLU	GIN	GLY	LEU	GLU	TRP	ILE	GLY	TYR	PIIE	SER	PRO	GLY	ASN	ASP
ASP	PIIE	LYS	TYR	ASN	GLU	ARG	PHE	LYS	GLY	LYS	ALA	THIR	LEU	THIR
ALA	ASP	LYS	SER	SER	SER	THIR	ALA	TYR	VAL	GIN	LEU	ASN	SER	LEU
THIR	SER	GLU	ASP	SER	ALA	VAL	TYR	PIIE	CYS	THIR	ARG	SER	LEU	ASN
MEI	<u>ALA</u>	<u>TYR</u>	<u>TRP</u>	<u>GLY</u>	<u>GIN</u>	<u>GLY</u>	<u>THIR</u>	<u>SER</u>	<u>VAL</u>	<u>THIR</u>	<u>VAL</u>	<u>SER</u>	<u>SER</u>	<u>SER</u>

"// indicates where the mature protein begins.
 ie underlined residues were determined by protein sequence. TRP can not be determined by the method used.

Figure 19

(AMINO ACID SEQUENCE OF CC-83 V₁₁)

MET	GLU	TRP	SER	TRP	VAL	PIIE	LEU	PIIE	LEU	PIIE	LEU	SER	VAL	THR	THR
GLY	VAL	HIS	SER	//GLN	VAL	GLN	LEU	GLN	LEU	GLN	SER	ASP	ALA	GLV	LEU
VAL	LYS	PRO	GLY	ALA	SER	VAL	LYS	ILE	LYS	SER	CYS	LYS	ALA	SER	GLY
TYR	THR	PHE	THR	ASP	HIS	ALA	ILE	HIS	ILE	TRP	VAL	LYS	GLN	LYS	PRO
GLU	GLN	GLY	LEU	GLU	TRP	ILE	GLY	TYR	GLY	ILE	SER	PRO	GLY	ASN	ASP
ASP	ILE	LYS	TYR	ASN	GLU	LYS	PHE	LYS	PHE	GLY	LYS	ALA	THR	LEU	THR
ALA	ASP	LYS	SER	SER	SER	THR	ALA	TYR	ALA	MET	<u>GLN</u>	<u>LEU</u>	<u>ASN</u>	<u>SER</u>	<u>LEU</u>
THR	SER	GLU	ASP	SER	ALA	VAL	TYR	PHE	TYR	CYS	ARG	ARG	SER	PIIE	
TYR	GLY	ASN	TRP	GLY	GLN	GLY	THR	THR	THR	LEU	THR	VAL	SER	SER	

25 / 48

"/" indicates where the mature protein begins.
 The underlined residues were determined by protein sequence.

26 / 48

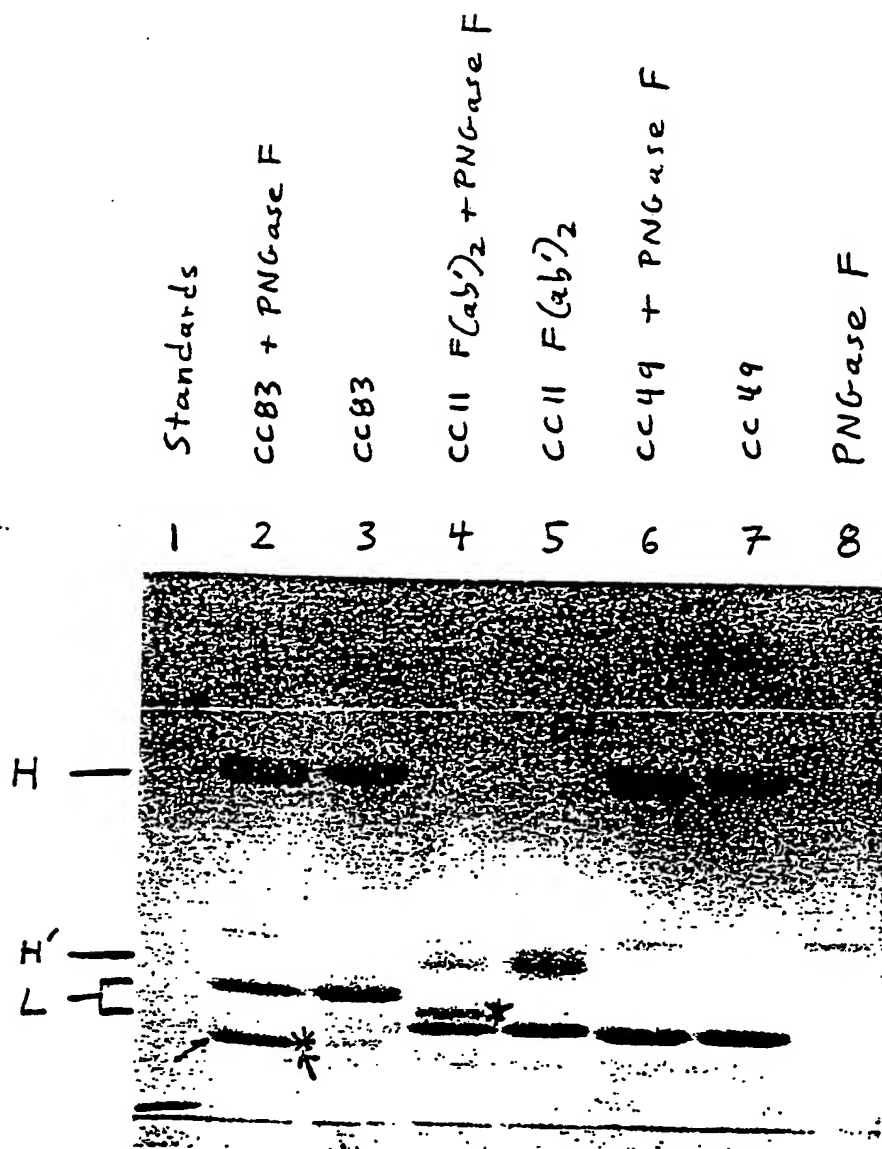


Figure 20

27 / 48

Human Heavy Chain Constant Regions

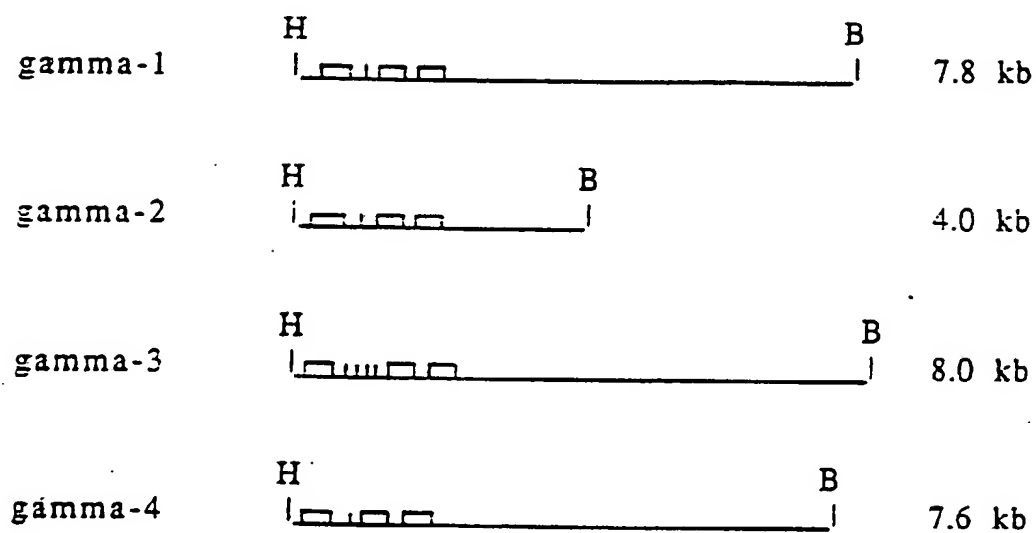


Figure 21

28 / 48

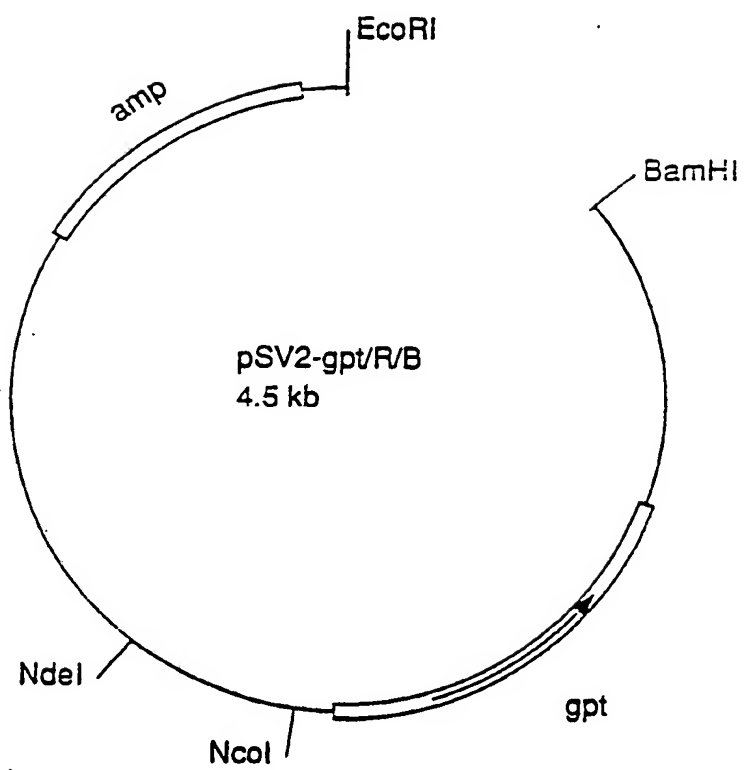


Figure 22

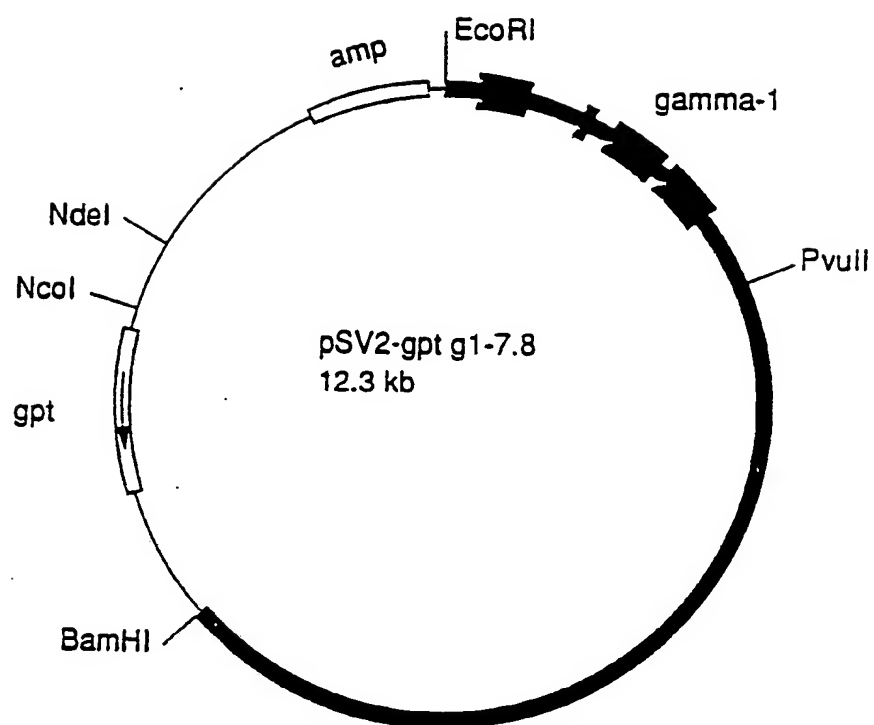


Figure 23 :

30 / 48

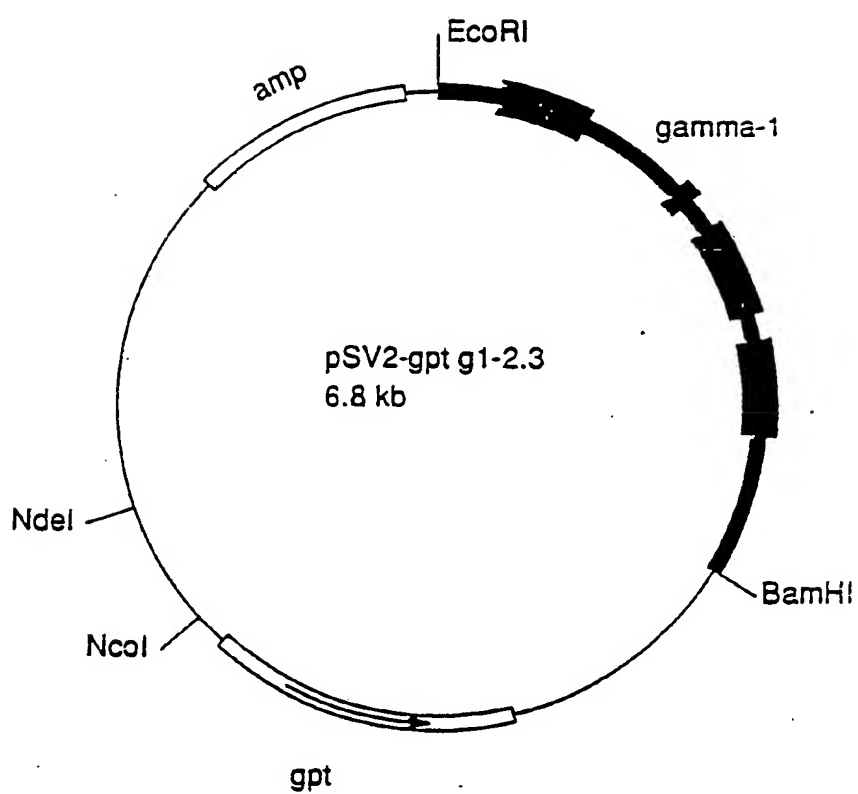


Figure 24

31 / 48

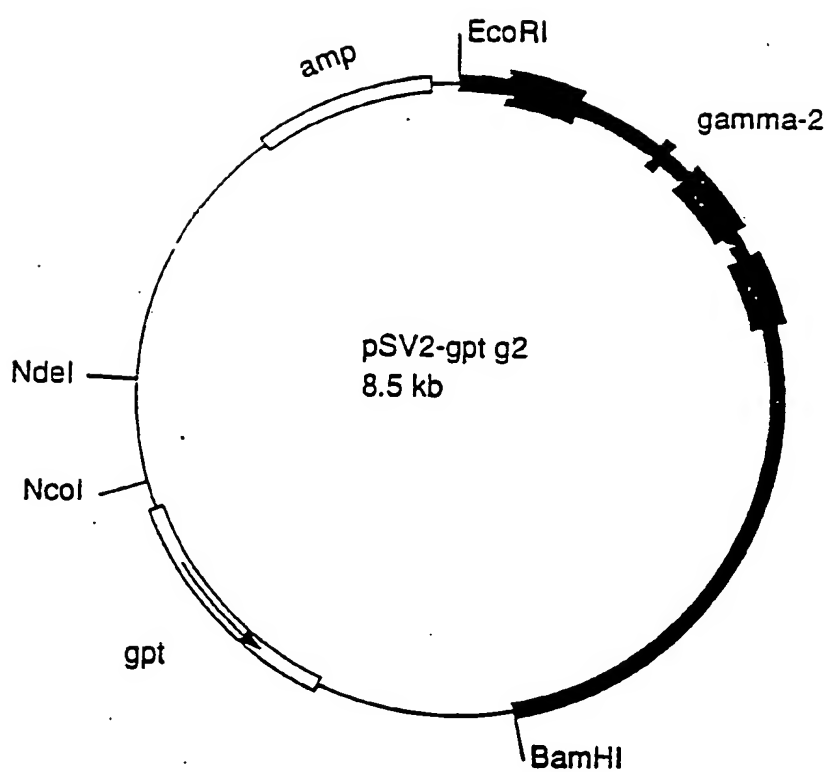


Figure 25

32 / 48

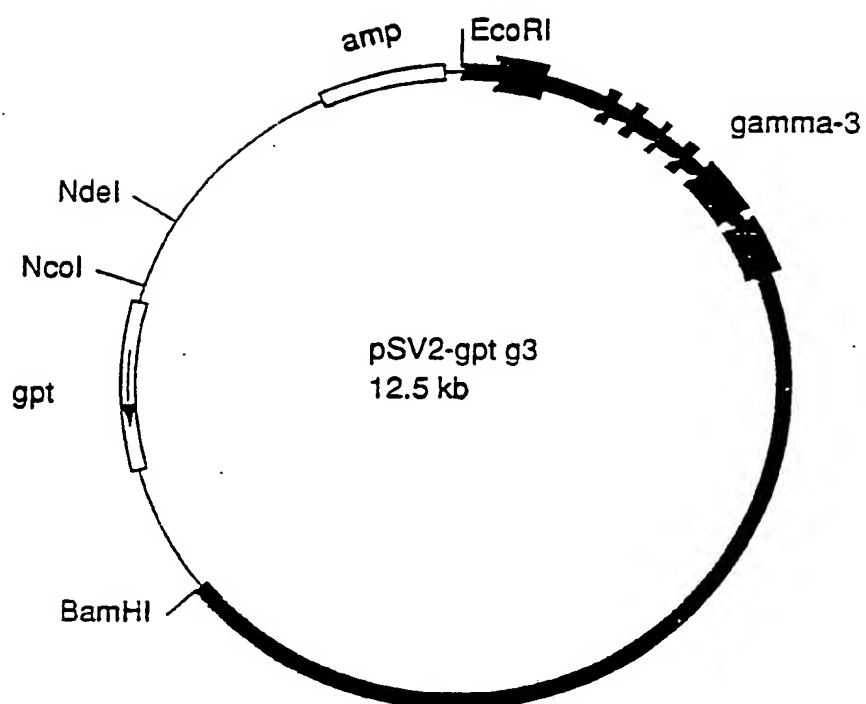


Figure 26

33 / 48

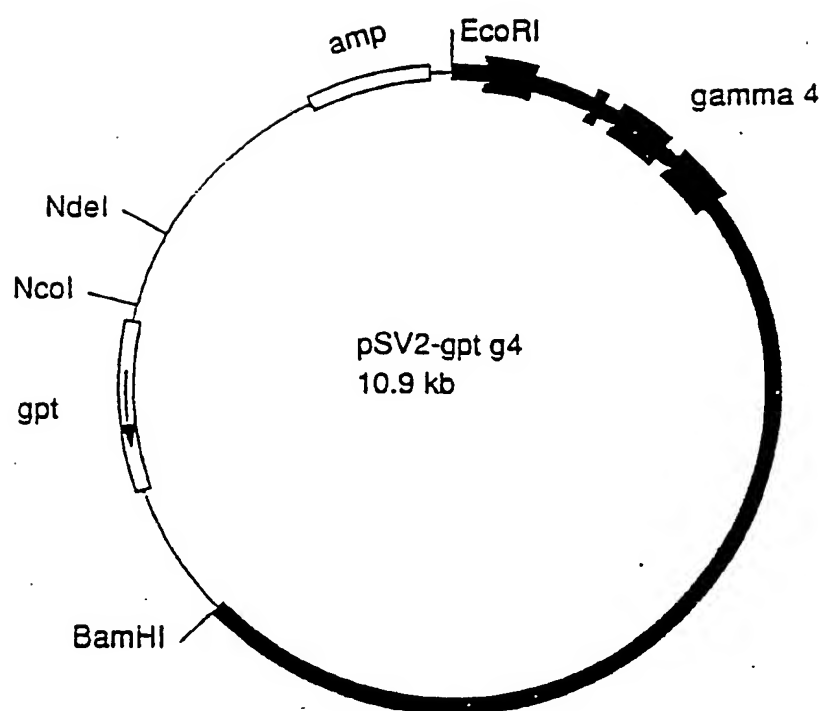


Figure 27

34 / 48

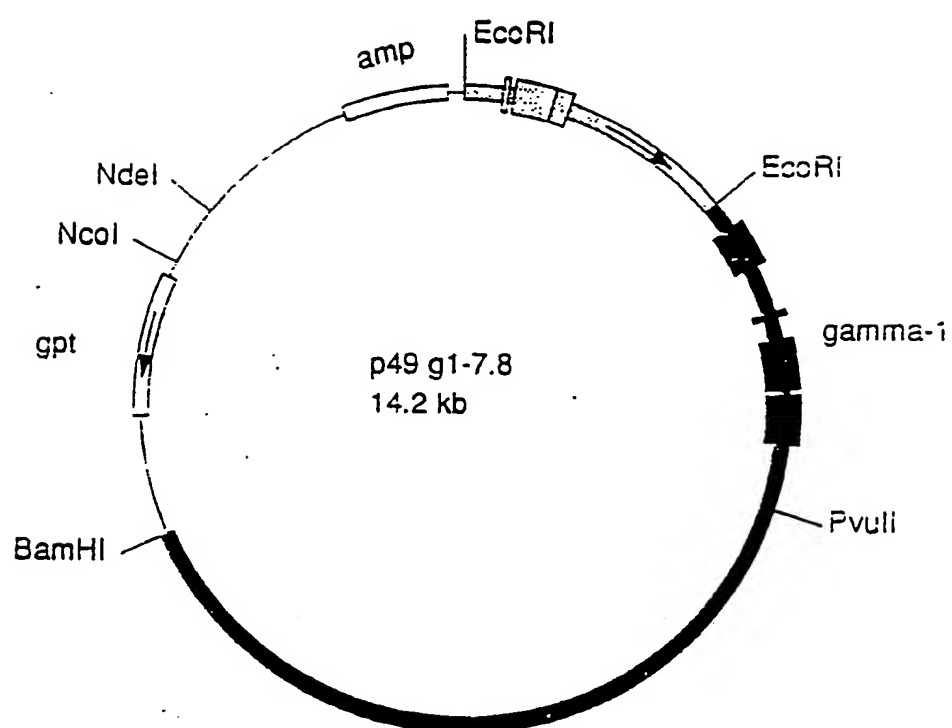


Figure 28

35 / 48

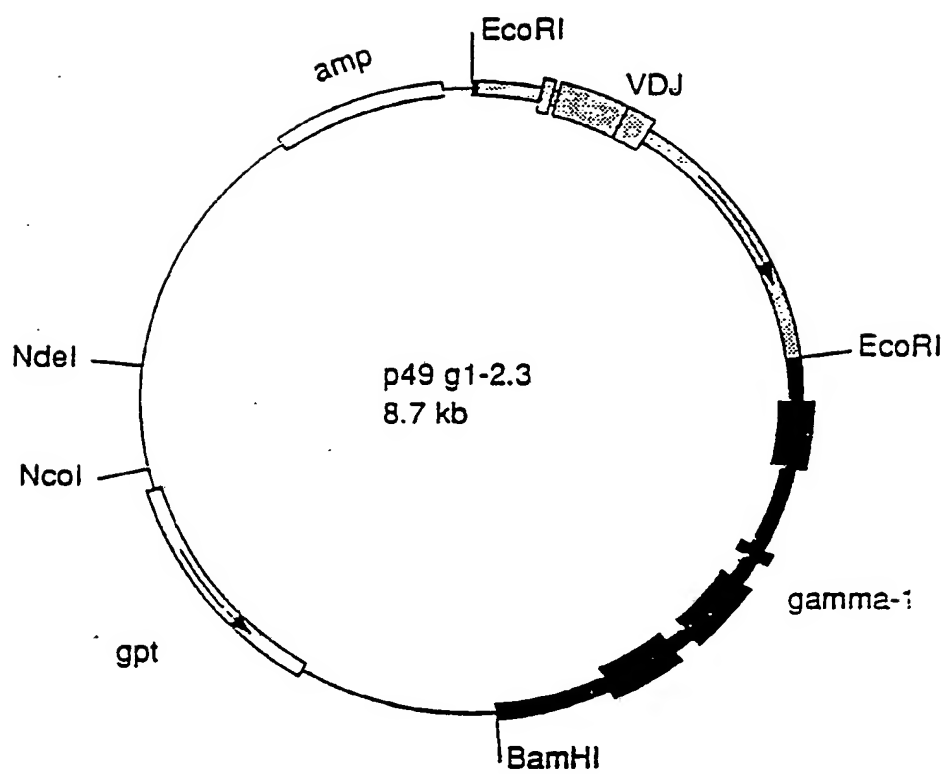


Figure 29

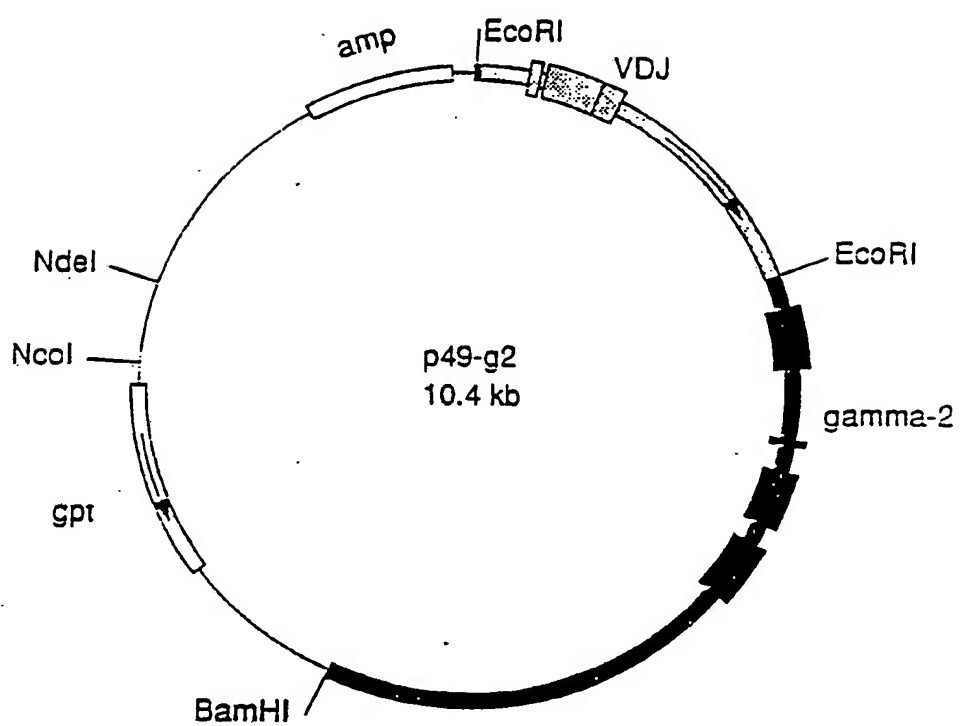


Figure 30

37 / 48

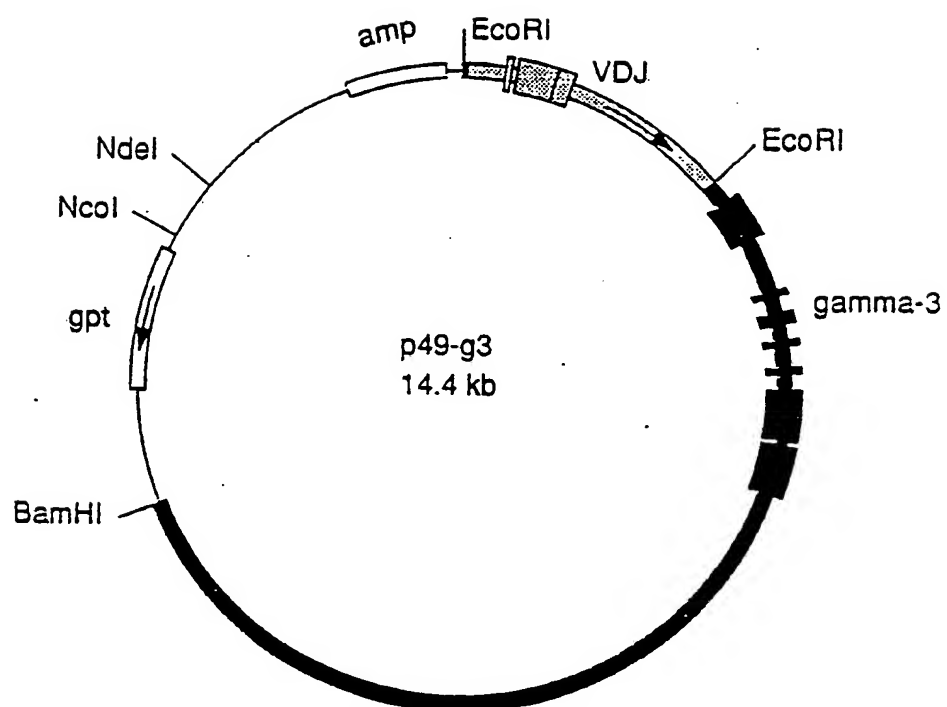


Figure 31

38 / 48

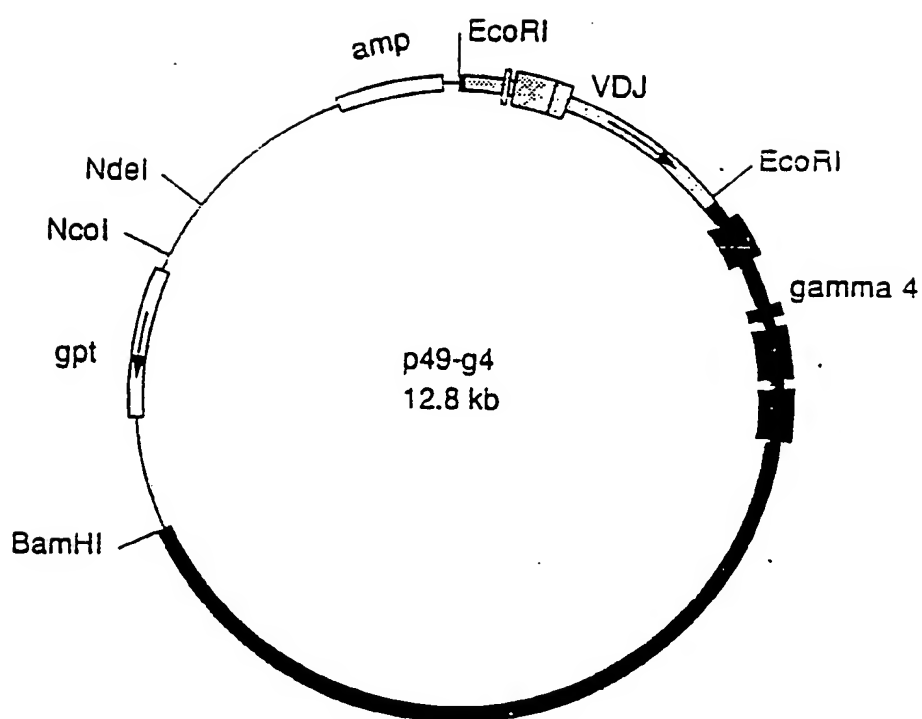


Figure 32

39 / 48

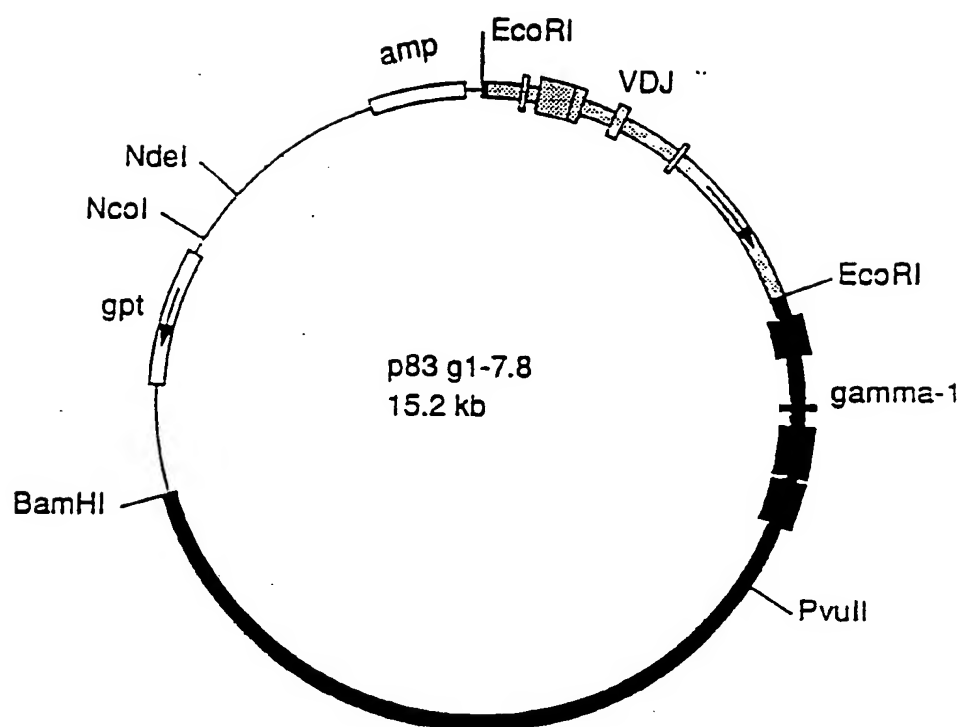


Figure 33

40 / 48

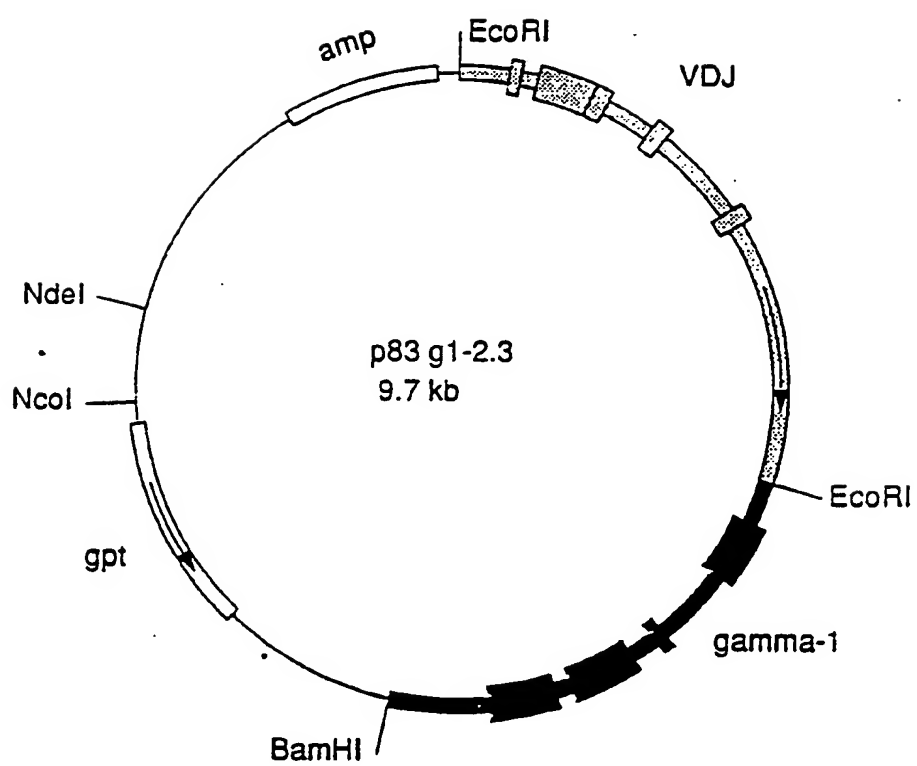


Figure 34

41 / 48

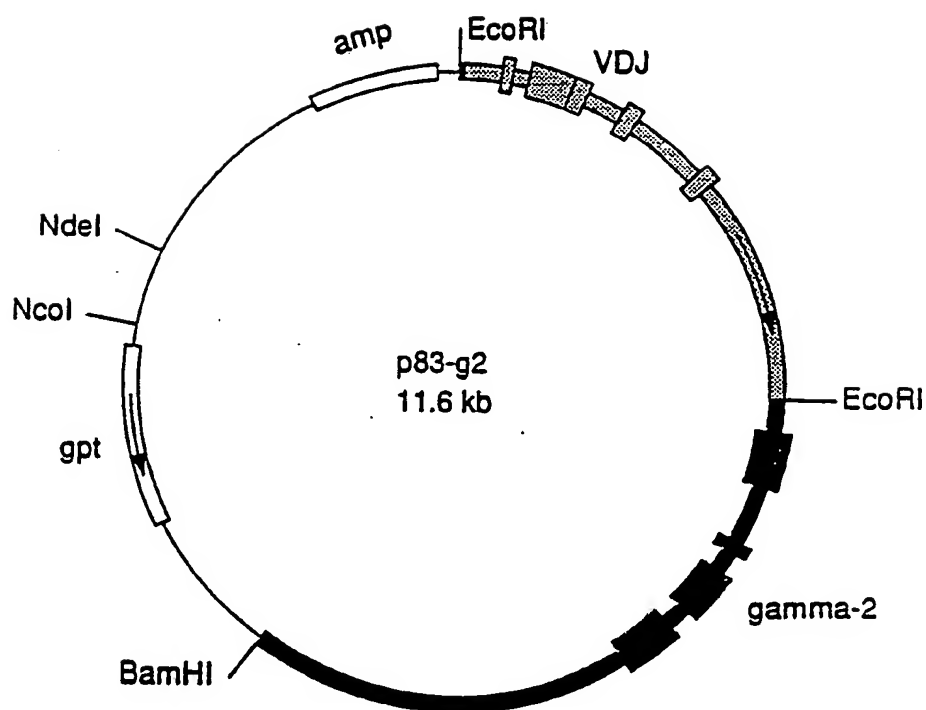


Figure 35

42 / 48

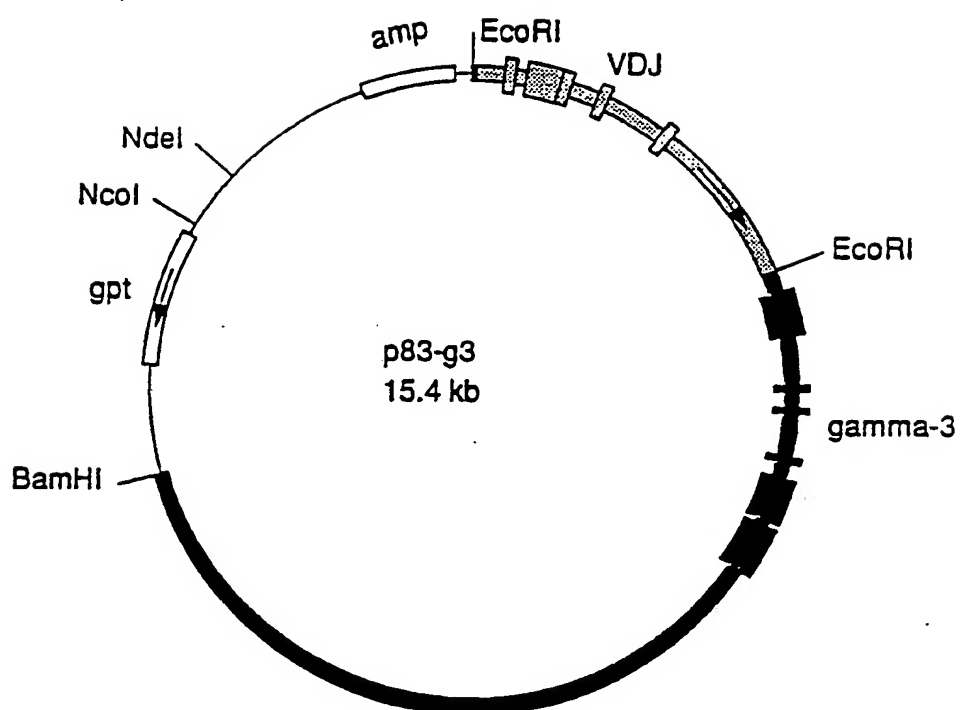


Figure 36

43 / 48

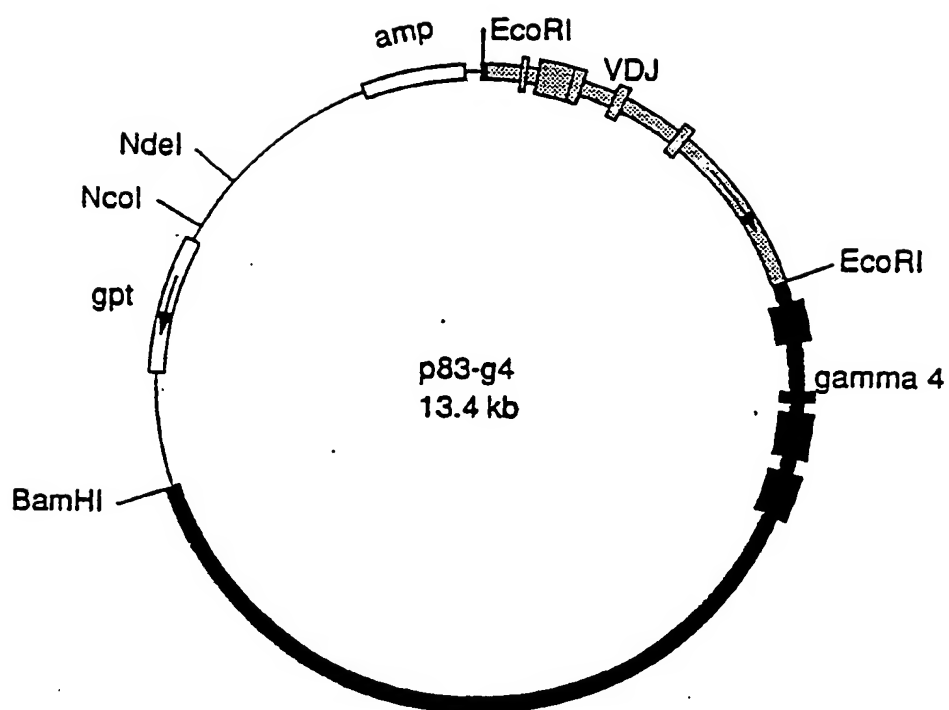
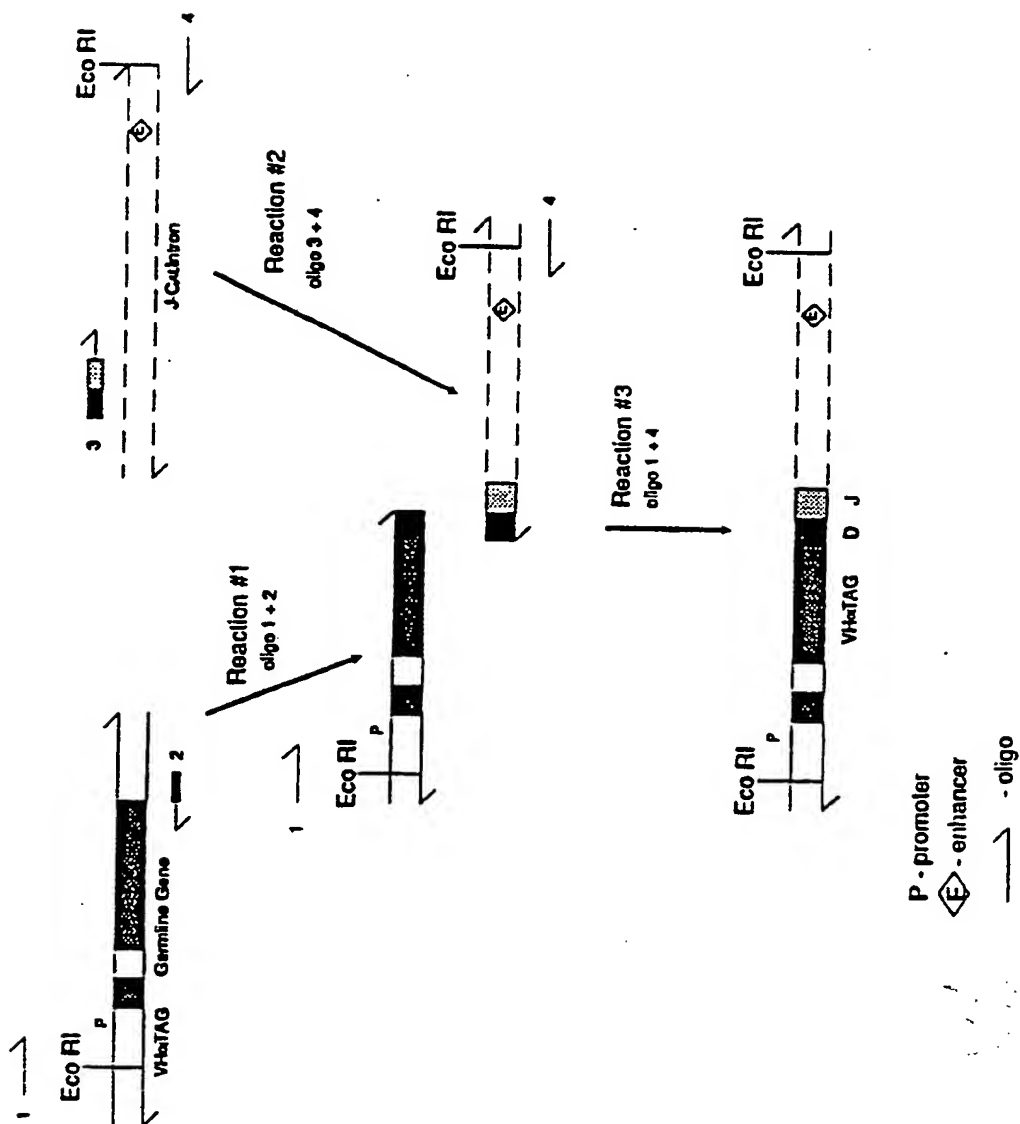


Figure 37

44 / 48

Figure 38



45 / 48

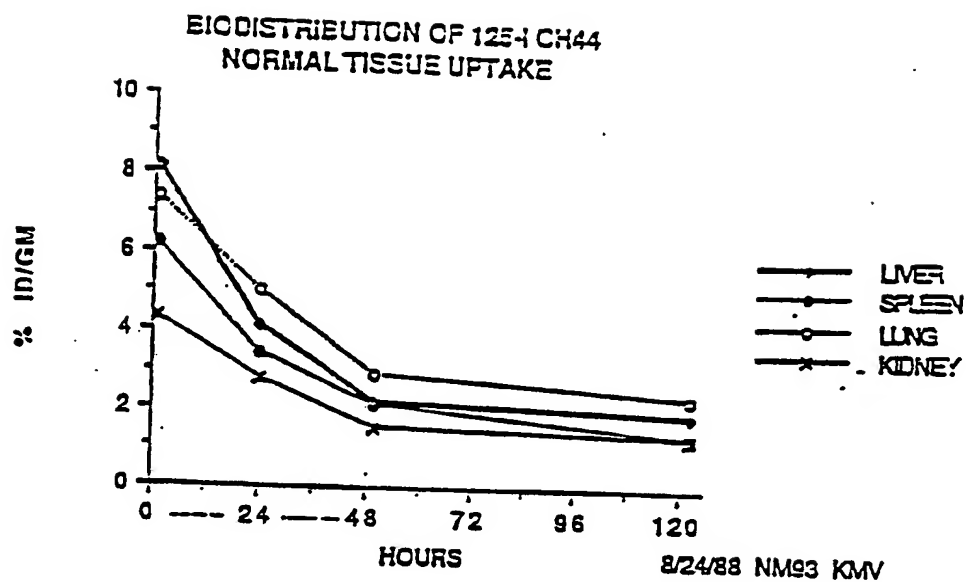


Figure 39a

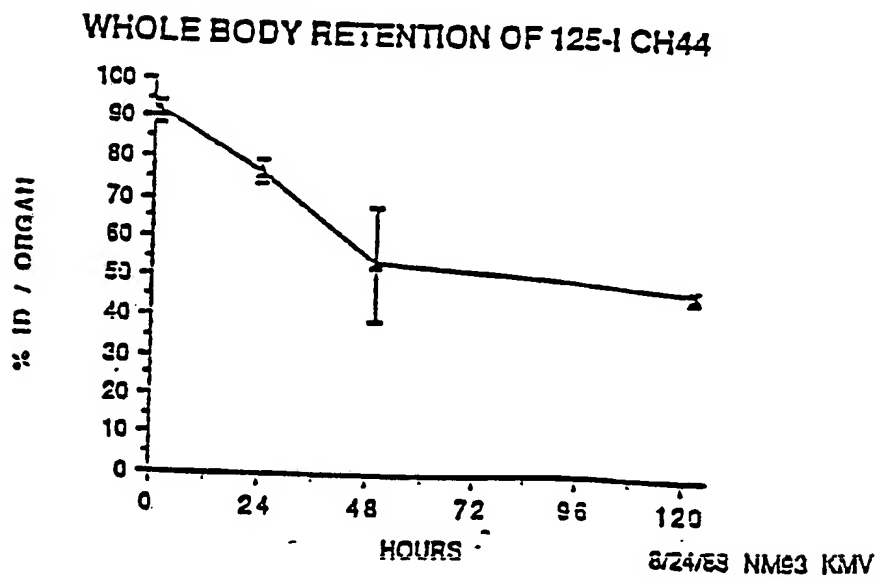


Figure 39b

46 / 48

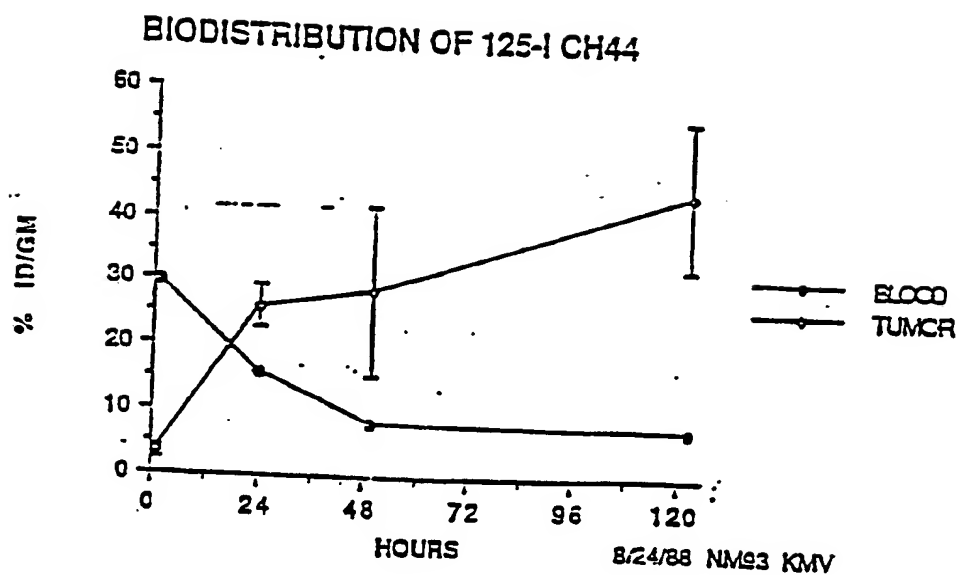


Figure 39c

47 / 48

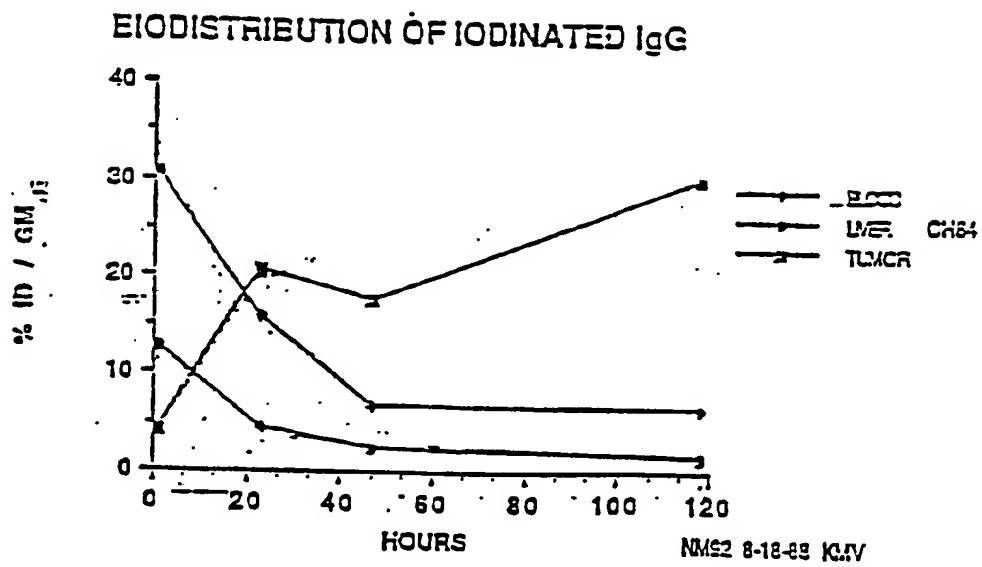


Figure 40a

48 / 48

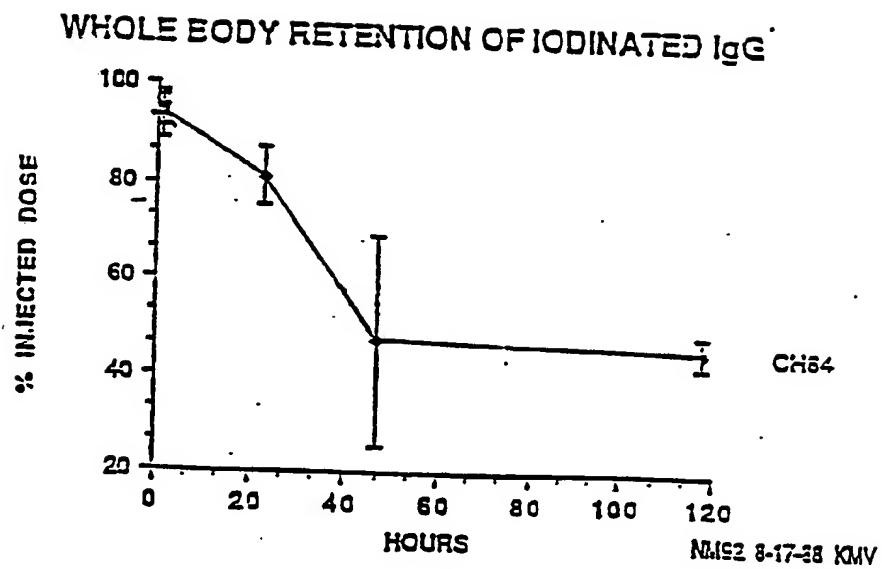


Figure 40b

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04402

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC I.P.C.(4): A61K 37/04, 39/00, 39/395, 39/44; C07H 15/12, 17/00 U.S.CL: 424/85.91, 85.8; 530/387, 389; 435/320; 536/27																	
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; text-align: left;">Classification System</th> <th style="border: 1px solid black; text-align: left;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">U.S.</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">424/85.91, 85.8 435/320; 530/387, 389; 536/27</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸</div> <p style="margin-top: 10px;">Automated Patent System, DIALOG Database searches. Search date 12/15/89.</p>			Classification System	Classification Symbols	U.S.	424/85.91, 85.8 435/320; 530/387, 389; 536/27											
Classification System	Classification Symbols																
U.S.	424/85.91, 85.8 435/320; 530/387, 389; 536/27																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border: 1px solid black; text-align: left;">Category [*]</th> <th style="width: 60%; border: 1px solid black; text-align: left;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; border: 1px solid black; text-align: left;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Proceedings of the National Academy of Sciences (USA), Vol. 81, pages 6851-6855. NOV 1984. (MORRISON), "Chimeric Human Anti-body Molecules. . ." See pp.6851, 6854.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-62</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Proc.National Acad. Sci.(USA), Vol.78(5), pp.3199-3203. MAY 1981 (COLCHER), "A Spectrum of Monoclonal Antibodies-Reactive with Human Mammary Tumor Cells". See p. 3200, Table 1.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-62</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">P,Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Cancer Research, Vol.49:1738-1745, APR 1989 (COLCHER), "Characterization and Biodistribution of Recombinant/Chimeric Constructs of Monoclonal Antibody B72.3." See entire article.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-62</td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="border: 1px solid black; vertical-align: top; padding: 5px;">Protein Engineering 1(6):499-505, DEC 1987. (WHITTLE), "Expression in COS Cells of a Mouse-Human Chimaeric B72.3 Antibody." See entire article.</td> <td style="border: 1px solid black; text-align: center; vertical-align: top; padding: 5px;">1-62</td> </tr> </table> <div style="margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> </div>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Proceedings of the National Academy of Sciences (USA), Vol. 81, pages 6851-6855. NOV 1984. (MORRISON), "Chimeric Human Anti-body Molecules. . ." See pp.6851, 6854.	1-62	Y	Proc.National Acad. Sci.(USA), Vol.78(5), pp.3199-3203. MAY 1981 (COLCHER), "A Spectrum of Monoclonal Antibodies-Reactive with Human Mammary Tumor Cells". See p. 3200, Table 1.	1-62	P,Y	Cancer Research, Vol.49:1738-1745, APR 1989 (COLCHER), "Characterization and Biodistribution of Recombinant/Chimeric Constructs of Monoclonal Antibody B72.3." See entire article.	1-62	Y	Protein Engineering 1(6):499-505, DEC 1987. (WHITTLE), "Expression in COS Cells of a Mouse-Human Chimaeric B72.3 Antibody." See entire article.	1-62
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³															
Y	Proceedings of the National Academy of Sciences (USA), Vol. 81, pages 6851-6855. NOV 1984. (MORRISON), "Chimeric Human Anti-body Molecules. . ." See pp.6851, 6854.	1-62															
Y	Proc.National Acad. Sci.(USA), Vol.78(5), pp.3199-3203. MAY 1981 (COLCHER), "A Spectrum of Monoclonal Antibodies-Reactive with Human Mammary Tumor Cells". See p. 3200, Table 1.	1-62															
P,Y	Cancer Research, Vol.49:1738-1745, APR 1989 (COLCHER), "Characterization and Biodistribution of Recombinant/Chimeric Constructs of Monoclonal Antibody B72.3." See entire article.	1-62															
Y	Protein Engineering 1(6):499-505, DEC 1987. (WHITTLE), "Expression in COS Cells of a Mouse-Human Chimaeric B72.3 Antibody." See entire article.	1-62															
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">21 DEC 1989</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">22 JAN 1990</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center;"> Thomas Cunningham, Ph.D </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">21 DEC 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">22 JAN 1990</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;"> Thomas Cunningham, Ph.D </div>											
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">21 DEC 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold; font-size: 1.2em;">22 JAN 1990</div>																
International Searching Authority <div style="text-align: center; font-weight: bold;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;"> Thomas Cunningham, Ph.D </div>																

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record .**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☒ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☐ **SKEWED/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.